Modulation of innate immunity by enteropathogen motifs

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DECLARATION

I, Aineias Spyridon Milioris, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Bacterial gastroenteritis affects millions, especially in the developing world. While the mechanisms by which enteric pathogens trigger inflammation have been studied extensively, how they evade immune recognition is less understood. We hypothesised that the structural components of common enteric pathogens, such as *Clostridium difficile* and *Campylobacter jejuni,* facilitate immune evasion by engaging inhibitory receptors on innate immune cells.

In the present study we investigated how *C. difficile* peptidoglycan (PGN) engages with monocytic cells and identified NOD2 as a critical innate sensor in this interaction. PGN affected cell maturation and in addition it modulated monocytic immune responses to other bacterial motifs, as PGN-exposed cells became unresponsive to subsequent Lipopolysaccharide (LPS) stimulation. This data suggests a role for *C. difficile* PGN-NOD2 axis in trained immunity.

We have previously reported on a novel interaction between *C. jejuni* flagellin pseudaminic acid moieties and the Siglec-10 receptor, an interaction that specifically targets the dendritic cell IL-10 axis. Herein, we studied the downstream signalling events involved in this crosstalk in human macrophages. Utilizing wild-type and isogenic mutant *C. jejuni*, we identified a potential role for the epidermal growth factor receptor (EGFR), the scaffolding SHP-2 and PI3K-AKT/MAPK kinase pathways in modulating IL-10 expression. We report for the first time novel engagement between *C. jejuni* 11168H capsule, lipooligosachharide (LOS) and host Siglec-5 and Siglec-9 receptors, pathways involved in bacterial adherence and phagocytosis.

Collectively, this thesis provides evidence as to how common bacterial structures exert immunosuppressive effects, effects that may hold the balance between asymptomatic colonization and infection.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PUBLICATIONS AND ABSTRACTS

Publications

Stephenson HN, Mills DC, Jones H, **Milioris E**, Copland A, Dorrell N, et al. Pseudaminic acid on Campylobacter jejuni flagella modulates dendritic cell IL-10 expression via Siglec-10 receptor: a novel flagellin-host interaction. Journal of Infectious Diseases. 2014 Nov 1;210(9):1487–98.

Abstracts

Milioris E, Iebba V, Klein NK and Bajaj-Elliott M (2013). Sodium butyrate modulates innate immunity to Enteropathogens. Front. Immunol. Conference Abstract: 15th International Congress of Immunology (ICI).

Milioris, E; Iebba, V; Klein, NJ; Steevels, T; Simmons, A; Bajaj-Elliott, M; (2012) The impact of NOD2 3020insC mutation in microbial-driven host innate immunity. Conference abstract: European Congress of Immunology (ECI).

ABBREVIATIONS

APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD domain
BSA	Bovine Serum Albumin
CARD	Caspase activating and recruitment domains
CFU	Colony Forming Unit
CHO	Chinese Hamster Ovary cell line
CPS	Capsular Polysaccharide
DAP	Diaminopimelic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular signal-regulated Kinase
FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GBS	Group B Streptococcus
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HEK	Human Embryonic Kidney cell line
HI	Heat inactivated
Ig	Immunoglobulin
IL	Interleukin
lgt	Lipoprotein diacylglyceryl transferase
JNK	c-Jun N-terminal kinases
LOS	Lipooligosaccharide
LPS	Lipopolysacchride
LRR	Leucine-rich repeat
M-CSF	Macrophage Colony Stimulating Facto
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
mDC	Monocyte-derived dendritic cell
MDM	Monocyte-derived Macrophage

MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NOD	Nucleotide oligomerisation domain
OD	Optical density
P	Probability
PBMC	Peripheral Blood-derived Monocytic Cells
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern Recognition Receptor
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SA	Sialic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SHP-2	Src homology 2 domain containing non-transmembrane PTP
Siglec	Sialic acid-binding immunoglobulin-type lectins
siRNA	Small-interfering RNA
STAT	Signal Transducer and Activator of Transcription
TEMED	Tetramethylethylenediamine
Th	T helper
THP-1	Tohoku Hospital Patient 1
TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor alpha
WT	Wild type

Chapter 1

Introduction

1.1 Gastrointestinal structure and function

The gastrointestinal (GI) tract includes all the digestive structures between the mouth and the anus. It is a complex and dynamic organ at the centre of an intricate interplay between a myriad of environmental stimuli and the host. Food ingested through the mouth passes through the pharynx and the esophagus and enters the stomach where it becomes digested. It then enters the small intestine, in the lower part of the GI tract, where it is broken down enzymatically in the duodenum followed by nutrient absorption in the jejunum and ileum. Water absorption takes place in the large intestine, also referred to as the colon, and rectum while the remaining waste material is expelled in the form of faeces.

The small intestine is the most important organ for digestion as it is where nutrient absorption takes place. To maximise the surface area available for nutrient absorption the epithelium of the small intestine is covered in finger-like protrusions called villi (Figure 1.1A). Additionally, each villus contains fine projections on its apical surface known as microvilli. Underlying the epithelium is the lamina propria (LP), which has a rich vascular and lymphatic network to absorb the digestive products. The epithelium around the villi contains crypts, moat-like invaginations lined largely with younger epithelial cells involved primarily in enzyme secretion.

The epithelium of the villi is made up of tall columnar absorptive cells called enterocytes, and goblet cells; the latter secrete mucin, for lubrication of the intestinal contents and protection of the epithelium (Figure 1.1B). The crypts contain Paneth cells, which have a defensive function, and various neuroendocrine cells involved in the production and secretion of hormones and neurotransmitters. Stem cells, found a few cells away from the base of the crypts, divide continuously to replace enterocytes, goblet cells, Paneth cells and neuroendocrine cells.

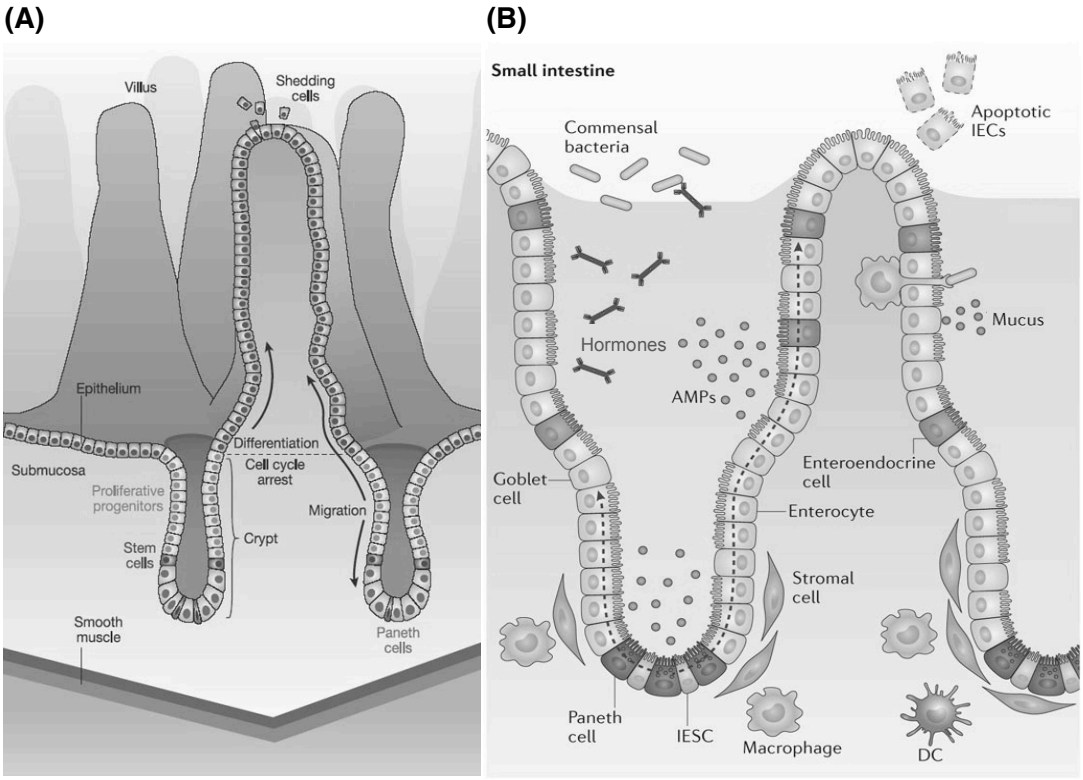

Figure 1.1. Structure and cells of the small intestine.

(A) Tissue anatomy of the small intestine. Putative stem cells reside above the Paneth cells near the crypt bottom. Proliferating progenitor cells occupy the remainder of the crypt. Differentiated cells populate the villus, and include goblet cells, enterocytes and entero-endocrine cells. (B) Intestinal epithelial cells (IECs) form a biochemical and physical barrier that maintains segregation between luminal microbial communities and the mucosal immune system. The intestinal epithelial stem cell niche, containing epithelial, stromal and haematopoietic cells, controls the continuous renewal of the epithelial cell layer by crypt-resident stem cells. Differentiated IECs migrate up the crypt–villus axis, as indicated by the dashed arrows. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial proteins (AMPs) to promote the exclusion of bacteria from the epithelial surface. Goblet cells mediate transport of luminal antigens and live bacteria across the epithelial barrier to DCs, and intestine-resident macrophages sample the lumen through transepithelial dendrites. [Adapted from ref. (1)]

1.1.1 Gastrointestinal immunity

The epithelium of the small intestine is in continuous contact with food antigens, commensal bacteria and potential pathogens (2). It is the organ tissue most exposed to the external environment with a surface area of 200m^2 densely populated by large numbers of microorganisms. There is a marked gradient of the numbers of commensal bacteria going down the intestine, from the almost sterile jejunum, to the descending colon, which has a large resident population of microbiota, consisting of at least 10^{10}-10^{12} organisms per gram of luminal contents (3). Continuous exposure to commensal and/or symbiotic microorganisms and their metabolites, along with the presence of pathogens and the antigenic load provided by diet, means that the GI mucosal immune system encounters more antigen/day than the systemic immune system encounters in a life-time. Not surprisingly, there is a strong biological necessity for a multifaceted, integrated epithelial and immune cell-mediated regulatory system for the maintenance of a healthy gut (4).

1.1.1.1 GI epithelial immunity

Consequently, the gut epithelium relies on multiple defense mechanisms, including a mucus layer, epithelial antimicrobial immunity, cell integrity and turnover. A single layer of intestinal epithelial cells (IECs) provides the distinct anatomical barrier that segregates the underlying tissues from potentially harmful compounds (5). The epithelial cells of the small intestine are coated in a glycocalyx of mucins and other glycoproteins that can interact with and entrap bacteria in the mucus so that they are simply washed away. In addition, anti-microbial peptides such as defensins are secreted by Paneth cells, providing another level of innate protection. IECs also act as

microbial sensors by secreting chemoattractant factors that facilitate the recruitment of a range of host immune cells (5).

1.1.1.2 GI mucosal immune system

In order to prevent translocation of commensals and, when present pathogenic microorganisms across this barrier, the intestinal tract possesses an integrated, highly sophisticated immune system. Intestinal immune cells located beneath the epithelium of both the small and large intestine play important roles in regulating the mucosal barrier and the resident microbiota. The majority of intestinal immune cells are located immediately below the epithelial layer in the lamina propria, either scattered or organised in tissue-specific lymphoid structures, such as Peyer's patches and mesenteric lymph nodes (MLN). The role of all these immunotypes is in the induction phase of intestinal immune responses, as they represent the sites where antigens are taken up and presented to B and T lymphocytes (6).

GI immunity is comprised of the innate and adaptive immune system. Innate cells include dendritic cells (DCs), monocytes, macrophages, neutrophils, and NK cells (7). Upon microbial sensing, these cells produce an appropriate inflammatory response, which includes the production of chemical mediators, pro- and anti-inflammatory cytokines and chemokines. The latter act as chemoattractants that prime innate immune cells, particularly neutrophils and inflammatory monocytes recruiting them to the site of infection (8). The adaptive immune system comprises immunoglobulin (Ig)-secreting plasma cells, B cells, $CD4^+$ and $CD8^+$ αβ-T cells, regulatory T (Treg) cells and γδ-T cells. The latter, intra-epithelial effector lymphocytes are integrated in the epithelial lining and extra-intestinal outposts, such as the MLNs and provide the host

with potent, adaptive immunity (9). The majority of mucosal T cells have a 'memory' phenotype and some show effector function such as cytokine production and cytotoxic T lymphocyte activity; others appear to be hyporesponsive and may be regulatory in nature (10).

1.1.1.3 Macrophages

Historically, macrophages were defined as tissue-resident innate immune cells with scavenging and bactericidal functions. Resident macrophages are found throughout the body and can have different morphology and immune specificity depending on their tissue of origin (11). These include Kupffer cells in the liver, microglia in the central nervous system, alveolar macrophages in the lung and the resident macrophages of the GI tract. All epithelial and endothelial surfaces contain a significant population of macrophages, generally located underneath the basement membrane separating the surface layer from the rest of the epithelial tissue. In the intestine, macrophages represent the most abundant mononuclear cell population, found mainly in the lamina propria of the small and large intestine (12).

Macrophages exhibit stellate morphology, and express a wide range of receptors, including those for the Fc portions of Ig, complement components and for microbial motifs known as microbe--associated molecular patterns (MAMPs). They also contribute to the non-specific uptake of particulate material which they degrade using enzymes such as non-specific esterase, lysosomal hydrolases and ecto-enzymes (7). When activated, tissue macrophages phagocytose and kill microorganisms, and secrete pro-inflammatory mediators. Amongst these are the chemokine interleukin-8 (IL-8), and the cytokines tumor necrosis factor alpha (TNFα), interleukins (IL) such as

IL-1β and IL-6. The pro-inflammatory cytokines and chemokines released upon activation contribute to the recruitment and activation of other innate immune cells, such as neutrophils, as well as antigen-specific lymphocytes (8). Macrophages can also instruct the adaptive immune system further, as they phagocytose, degrade and process antigens for presentation to T cells on MHC molecules, and may even be able to prime naïve T cells in vivo (13).

1.1.2 Gastrointestinal (GI) homeostasis

The GI mucosal immune system relies to a great degree on innate immune cells and their rapid effector functions for early sensing of both commensal and pathogens and the maintenance of tissue homeostasis. Signals from commensal bacteria can influence immune cell development and susceptibility to infectious or inflammatory diseases. According to studies, commensal-derived compounds offer a degree of sustained, tonic stimulation that potentiates the immune cell response and effective clearance of bacterial and viral pathogens, acting as a rheostat for the activation threshold of these cells during immune-surveillance. For example, mice are less likely to develop infection-induced colitis in the presence of bacterial peptidoglycan (14). In turn, innate immune cells act as key modulators of commensal flora composition and homeostasis; maintaining the equilibrium known as intestinal tolerance (1).

1.1.2.1 Inflammatory Bowel Disease

Inappropriate responses against innocuous food and commensal antigens can lead to inflammatory disorders such as coeliac disease and inflammatory bowel diseases (IBDs). The two main forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC),

are chronic, relapsing inflammatory disorders that result in loss of intestinal architecture and tissue destruction. UC is restricted to the colon and involves a superficial inflammation, whereas CD can affect the entire GI tract and is characterised by transmural inflammation and the formation of macrophage-rich granulomas. Both forms of IBD involve a substantial infiltrate of neutrophils, monocytes, eosinophils and T cells into the intestine (9).

1.1.2.2 Infection

Enteropathogenic infections can also lead to disruption of GI homeostasis, which can manifest in a range of clinical symptoms: from watery, bloody diarrhoea to colitis. Infection by *Campylobacter jejuni* is considered to be the most prevalent cause of bacterial-mediated diarrhoeal disease worldwide. *Campylobacter* infection causes local acute inflammatory changes in both the small and large bowel (15). Bacterial adhesion and invasion of the intestinal epithelium is succeeded by initiation of the inflammatory processes and diarrhoeal development. A steady rise in reported cases over the years has been attributed to the extensive colonization of livestock as well as antibiotic resistance (European Food and Safety Association; EFSA). *Clostridium difficile* is another major causal agent of antibiotic-associated diarrhea and is a leading cause of hospital-acquired infections. *C. difficile* has been known to cause severe diarrhea and colitis, but the emergence of newer, hypervirulent strains of *C. difficile* has further compounded the problem, and recently both number of cases and mortality associated with *C. difficile* infection (CDI) has markedly increased into a serious global health burden (16). Although CDI affects mostly individuals with compromised immunity, such as the elderly or patients on antibiotic therapy, cases are also emerging in the community and in animals used for food (17).

1.2 Innate immunity to bacterial motifs

To survey and regulate the GI ecosystem effectively, innate immune cells need to distinguish between potentially pathogenic microbial components and harmless antigens. Conserved structures found in microbes but not in the mammalian host (MAMPs) are recognized by cellular sensors known as pattern-recognition receptors (PRRs) (18). The two major families of PRRs are the cell surface and endosomal Toll-like receptors (TLRs) and the cytoplasmic nucleotide-binding domain, leucine-rich repeat (NLR) proteins. These receptors are highly expressed on professional antigen presenting cells (APCs) such as DCs and macrophages.

1.2.1 Toll-like receptors

Toll-like receptors (TLRs) were first identified in Drosophila (19) with a human ortholog discovered subsequently (20). TLRs are type-1 transmembrane proteins, comprised of a C-terminal leucine-rich repeat (LRR) domain that is ligand-binding, a single membrane spanning domain, and an N-terminal cytoplasmic Toll/IL-1R (TIR) domain that binds to adaptor molecules to trigger downstream signalling cascades (21). There are 11 TLRs known in mammals and each recognises MAMPs associated with a unique category of microbial antigens. Most TLRs are present on mononuclear immune cells, either on the cell surface or within endosomes (22). TLRs have also been detected on B and T cells (23),(24). TLR4 was the first to be discovered and recognises lipopolysaccharide (LPS), a structural component of Gram-negative bacteria (25).

Early studies identified peptidoglycan (PGN) as the TLR2 agonist (26), although this was soon disputed by evidence of the presence of TLR2-activating lipoteichoic acid (LTA) components in cell wall extracts (detailed in section 1.3.1.4.3) (27). Yet, further research employing a lipoprotein negative mutant of *Staphylococcus aureus* showed that surface lipoprotein rather than LTA or PGN activate TLR2 (28). Until recently, lipoproteins were thought to be the only bacterial wall ligands sensed by TLR2 at physiological levels (29). However, a recent study showed that Gram-positive bacterial lipoglycans, whose lipid anchor is made of a glycosylated diacylglycerol unit, are also recognised by TLR2 (30). Unlike other TLRs, which are functionally active as homo-dimers, TLR2 can heterodimerise with TLR1 or TLR6 to distinguish between Gram-negative tri-acylated lipoproteins and Gram-positive di-acylated lipoproteins respectively (31).

TLR activation by MAMPs leads to the activation of multiple signalling cascades; mediated by a number of scaffolding proteins that contain TIR-containing adaptor molecules, such as myeloid differentiation primary response protein (MyD88), MyD88 adaptor-like (Mal), TIR domain containing adaptor inducing INF-β (TRIF) and TRIF related adaptor molecule (TRAF). TLR2-driven signalling cascades result in the activation of MAPK, NF-κB and interferon regulatory factor (IRF) pathways which are central to the cell's inflammatory response (32). TLR2 activation leads to the NF-κB-dependent induction of IL-8 (26). It also drives IL-6 through MAPK signalling (33). Furthermore, TLR2 signalling activates autophagy, a key cellular process involved in processing of bacterial MAMPs, via ERK activation (34). Interestingly, while heterodimerisation of TLR2 with TRL1 or TLR6 expands the ligand spectrum it does not lead to differential signalling (31). PGN containing lipoproteins can induce maturation of blood cells by inducing the expression of CD14, TLR2 and TLR4 (35). Furthermore, the

TLR2 axis is seen as a priming factor for LPS signalling since it can modulate the LPS-mediated cytokine response in human monocytes. The synergistic effect is mediated by IRAK4, a shared signalling mediator, leading to the enhanced secretion of pro-inflammatory cytokines such as TNF α. Researchers are looking to exploit this property of TLR2 ligands in vaccine development as they are effective adjuvants (36).

1.2.2 Nod-like receptors

To date, 22 nucleotide oligomerisation domain (Nod)-like receptors (NLRs) have been identified in humans and 34 in mice, found primarily in immune and epithelial cells (37). NOD1 is widely expressed in cells of haematopoietic and non-haematopoietic origin. NOD2 expression is more restricted and the protein is mostly found in the haematopoietic lineage in myeloid and lymphoid cells. NOD1 and NOD2 were the first members of the NLR family to be discovered (38),(39) and the first NLRs to be identified as MAMP sensors. Despite being initially identified as receptors for bacterial LPS, further refinement and purification of bacterial cell preparations revealed that the NOD proteins recognise bacterial PGN (40).

1.2.2.1 Structure

Members of the NLR family are characterized by a tripartite domain structure, including a functionally differentiating domain distinct in each of the four subfamilies. The NOD1 and NOD2 proteins consist of a central nucleotide-binding domain (NBD; also known as NOD domain) which contains NACHT, winged helix and superhelical subdomains involved in oligomerisation and activation (41). The carboxy-terminal leucine-rich

repeats (LRRs) are important for ligand-sensing, while the N-terminus caspase recruitment domain (CARD) constitutes the effector domain and dictates the interactions of the receptor with different downstream effector molecules (42). NOD1 is composed of a single CARD, whereas NOD2 contains two CARD domains in tandem. Ligand recognition is thought to lead to a conformational change that results in NBD-mediated oligomerisation of the NOD proteins. Subsequently, NOD1 or NOD2 oligomers recruit the downstream adaptor RICK through CARD-CARD interactions forming a large signalling assembly (39),(43),(44).

1.2.2.2 NOD signalling

Each of the NOD receptors recognises a distinct PGN moiety. NOD2 recognises MDP, the PGN-derived muropeptide a moiety common to the cell wall of all Gram-positive and Gram-negative bacteria (45),(40). NOD1 recognises DAP found in the PGN of Gram-negative and some Gram-positive bacteria (46). Despite their discovery over a decade ago, direct binding of these motifs to the NOD receptors was only recently demonstrated. The binding interaction between NOD1 and DAP was shown to require the LRR domain (47). In contrast, the interaction of NOD2 with MDP was mediated by the central NBD rather than the LRR domain (48).

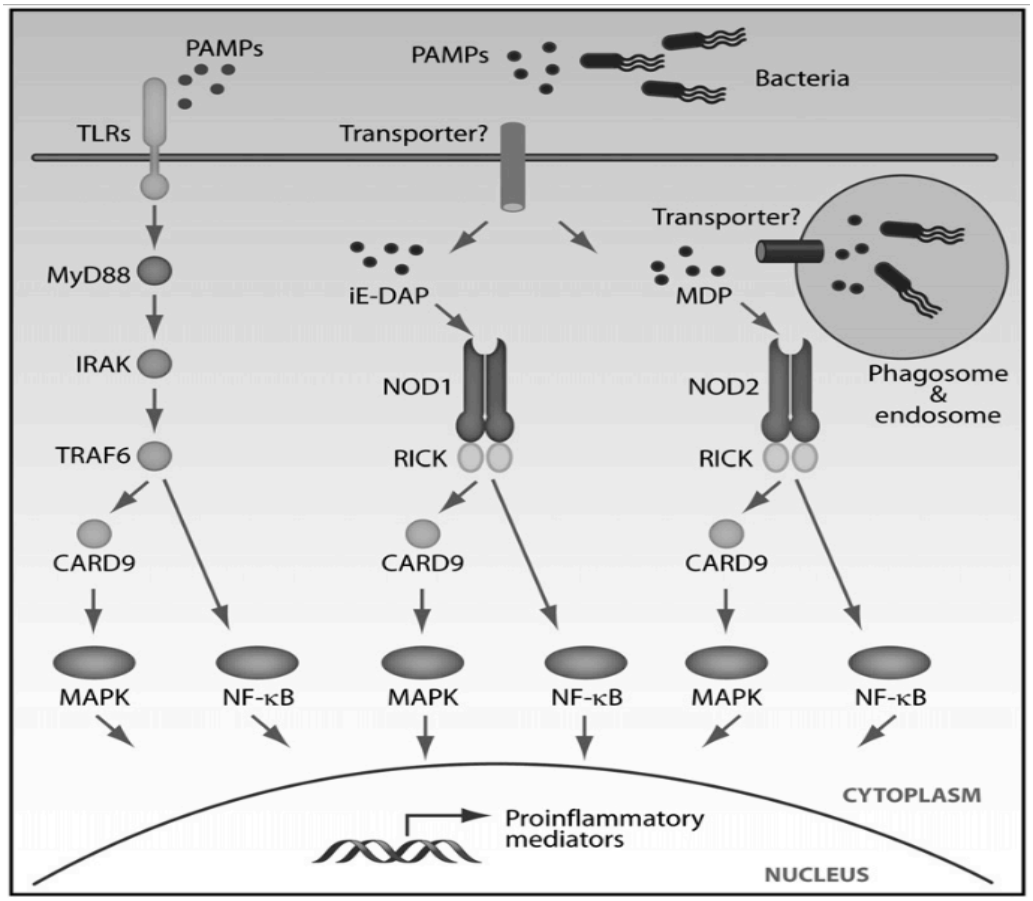

Figure 1.2. Model for PGN recognition by NOD1 and NOD2.

The NLR proteins NOD1 and NOD2 sense intracellular DAP and MDP, respectively, leading to recruitment of the adaptor proteins RICK and CARD9. Extracellular PAMPs are recognized by TLRs, which signals through MyD88, IRAK proteins, and TRAF members. The subsequent activation of NF-κB and MAP kinases results in the transcriptional upregulation of proinflammatory genes. [Adapted from ref. (49)]

In the absence of NOD ligand, the NACHT domain is inhibited through interaction of the C-terminal LRR fragment with the N-terminal CARD-NACHT fragment. Upon activation by DAP or MDP respectively, the NOD proteins undergo conformational changes allowing the NACHT domain to self-oligomerise (50). Subsequently, exposure of the CARD fragment recruits RICK (RIP2), a CARD-containing serine threonine kinase, via CARD/CARD interactions (51) (Figure 1.2). Activated RIP2 triggers two distinct downstream pathways; NF-κB and MAPK signalling. RIP2 activates IKK, either directly or by recruitment of the transformation growth factor β-activated kinase (TGF-β-activated kinase 1 (TAKI), leading to the phosphorylation and subsequent degradation of IκB resulting in the release and nuclear translocation of NF-κB (39),(51), a master transcriptional regulator of inflammation. Rip2 also activates the p38 and Erk MAP kinases upon MDP stimulation, although this interaction is less well-characterized (52). These pathways culminate in the induction of proinflammatory cytokines, chemokines and host defence.

In addition to this canonical signalling, downstream effectors of NOD2 have been implicated in regulation of the anti-inflammatory cytokine IL-10. IL-10 is a pleiotropic cytokine involved in immunoregulation and dampening of inflammation. RICK is required for NOD2-mediated IL-10 production (53), but is only part of an intricate signalling network. Furthermore, NOD1 and NOD2 activation also induces cross-talk between the signalling factor Notch and members of the phosphoinositide-3-kinase (PI3K) pathway which controls the expression of IL-10 (54),(55). Accordingly, CD - associated mutations in NOD2 lead to a reduction in IL-10 transcription (56). Remarkably, this was not due to a loss-of-function but rather active inhibition of the hnRNP-A1 transcriptional controller of IL-10 by the mutant NOD2 protein (57). Despite the extensive effort that has gone into deciphering the role of NODs in immune signalling, a great deal of controversy regarding their function remains to this day. It is

worth noting that mutations in NOD2 often leads to opposite functional outcomes in mice compared to humans (58) which has led to numerous conflicting reports (59).

1.2.2.3 NOD - TLR crosstalk

The NOD receptors share signalling pathways with TLR2, the cell surface receptor for PGN and TLR4, which binds to LPS. NODs act synergistically with TLR4 signalling to enhance the immune response to LPS (60),(61). A study found that PGN co-localises with both TLR2 and NOD2 to induce innate immune signalling in macrophages (62). The synergistic action between TLR2 and NOD2 is important in the production of not only pro- but also anti-inflammatory immunity in response to Gram-positive cell walls (53). NOD1 and NOD2 are thought to enhance the TLR-mediated response in monocytes, NK cells (63) and IECs (64). Crosstalk has also been reported for non-canonical signalling, as TLR2 activation enhances NOD-mediated Notch/PI3K signalling (55). In T cells however, NOD2 acts as a negative regulator of TLR2-mediated Th1 type responses and might explain how mutations in NOD2 lead to excessive inflammation (65). Since the complex cross-talk amongst these receptors is pivotal in the maintenance of a balanced pro-/anti-inflammatory axis, it is important that these interactions are studied in concert.

1.2.2.4 Outcome of NOD activation

NOD-mediated sensing of PGN has been implicated in activating the immune system in many species (66),(67),(68). Amongst the pro-inflammatory immune factors are TNFα, IL-6, CC-chemokine ligand (CCL2), IL-8 and antimicrobial factors such as defensins (69),(70). Besides mediating the inflammatory response, NOD2 activity has also been linked to IL-10 (71). Furthermore, NOD activation leads to the transcription of

type I interferon (IFN) genes that are more commonly associated with viral infection (72). Although the focus of this thesis is on bacterial-innate immune crosstalk, it is worth noting that NOD proteins are also important in adaptive immunity as they are involved in the priming of T-helper cells and drive T helper 2-type immunity (73).

1.2.2.5 NODs in immune homeostasis

1.2.2.5.1 Pathology

NOD2 was identified as a susceptibility gene for CD, a chronic inflammatory disorder of the GI tract that affects mainly the terminal ileum, cecum, colon and perianal area. Disease pathogenesis is thought to arise from a loss-of-function mutation leading to deregulated crosstalk between resident microbiota and the mucosal immune system (74). On the other hand, gain-of-function mutations in the NBD region of NOD2 lead to auto-inflammatory diseases such as Blau syndrome and early onset sarcoidosis. More recently, defects in NOD2 PGN sensing has been linked to diet induced inflammation and insulin resistance (75). Despite the homology in protein structure and shared downstream signalling only gene variants of NOD2, but not NOD1, have been linked to disease.

1.2.2.5.2 Bacterial sensing

Both NOD1 and NOD2 are cytoplasmic proteins that become recruited to the plasma membrane where they detect invading bacteria. The first study to show NOD activation by a microorganism was conducted using *Shigella flexneri* (76); an invasive enteropathogen, which is consistent with their role as intracellular sensors. Since then, murine studies have demonstrated that NOD1 or NOD2 deficiency does indeed increase susceptibility to bacterial infection. NOD1 has been linked with the immune response against *C. difficile* (77), *Campylobacter jejuni* (78), *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (59). The pathogenic bacteria recognised by NOD2 are *Streptococcus pneumoniae, Mycobacterium tuberculosis, Staphylococcus aureus, L. monocytogenes, Salmonella serotype* typhimurium*, Bacillus anthracis* and more (59). Although a pathogen may be recognised by only one of the two receptors, they both cooperate in the innate immune response to enteric pathogens. For example, In a study in knock-out mice infected with *Salmonella,* only mice lacking both NOD proteins exhibited increased bacterial burden in the mucosal tissue (79).

1.2.2.5.3 Immune tolerance

Despite continual exposure to high levels of resident microbiota the GI mucosal immune system is able to maintain a homeostatic environment. One mechanism contributing to this is the relative tolerance of intestinal resident macrophages to bacterial products, which are instructed to become tolerant on entering the intestine (80). Unlike peripheral macrophages, intestinal macrophages do not secrete cytokines on stimulation of PRRs or cytokine receptors, indicating that they are already tolerant to

these stimuli (81). However, this down-regulation is impaired in individuals with CD-associated NOD2 polymorphisms (82), leading to the hypothesis that tonic stimulation with NOD ligands is essential for the maintenance of intestinal equilibrium. Accordingly, Hedl and Abraham have shown that chronic, as opposed to acute, NOD2 stimulation down-regulates pro-inflammatory cytokines on re-stimulation with MDP leading to development of “self-tolerance” in monocyte-derived macrophages (MDMs) (81). Furthermore, chronic NOD2 stimulation leads to a diminished inflammatory response to TLR2 and TLR4 ligands, termed “cross-tolerance” (83). This is effected through NOD2-dependent epigenetic reprogramming of gene promoter regions (84). The mechanism involves the release of nitric oxide following NOD1 or NOD2 stimulation (55) and activation of Notch1-PI3K signalling (54) that drives the reprogramming of the macrophages. Interestingly, while macrophages become insensitive to TLR ligands they remain responsive to NOD1 and NOD2 stimulation and NOD-mediated signalling and gene expression are enhanced (85).

1.2.2.5.4 GI homeostasis

Stimulation of NOD1 and NOD2 by the microbiota is important for gut homeostasis. Microbial ligands enter the cytosol of IECs inducing low-level tonic signalling via NOD activation. This drives the release of antimicrobial factors that maintain the apical surface of the epithelium relatively sterile. PGN released from commensal bacteria offers protection from induced colitis in a mouse model in a NOD2-dependent manner (4). In 2009, Petnicki-Ocwieja et al., identified a potential new role for NOD2 in GI homeostasis by noting that a lack of NOD2 or RICK resulted in an increased load of certain commensal bacteria in the terminal ileum (86). CD patients with NOD2

polymorphisms exhibit defects in bacterial clearance from ileal crypts of the intestine (87). A few studies to date, point to bi-directional homeostatic regulation between NOD2 and commensal bacteria; a feedback loop in which gut-residing bacteria positively regulate NOD2 and its associated signalling molecules, which in turn negatively regulate the commensal flora (88). NOD2 also has a role in regulating the effector functions of GI mononuclear phagocytic populations by inducing chemoattractants that recruit these cells, which in turn promote bacterial clearance (89).

1.2.2.6 Inflammasomes

Other members of the NLR family are also involved in sensing MAMPs. NLRC4/Ipaf is activated by bacterial flagellin (90), mouse Nalp1b by lethal toxin produced by *B. anthracis* (91), NLRP3/cryopyrin is activated in response to a variety of microbial molecules (92), as well as endogenous ligands, such as uric acid crystals (93). Upon activation, these NLRs form the inflammasomes, large molecular assemblies responsible for processing pro-caspase-1 into the enzymatically active heterodimer caspase-1. Active caspase-1 cleaves the precursor pro-IL1β and pro-IL-18 into mature bioactive IL-1β and IL-18. NLRP3 and NLRC4 inflammasomes also require the adaptor protein ASC [apoptosis-associated speck-like protein containing a CARD) in order to activate caspase-1 and process IL-1β and IL-18. These events also result in pyroptosis, a specialised form of inflammatory cell death (distinct from apoptosis and necrosis) following bacterial infection. Induction of pyroptosis in macrophages and DCs initiates the release of pro-inflammatory cytokines, growth factors, endogenous danger-associated molecular patterns (DAMPs) and microbial antigens (94).

Currently, the contribution of NOD2 ligands to inflammasome activity is unclear. In 2008, M. Karin and colleagues showed direct interaction of NOD2 to caspase-1 via a CARD-CARD interaction, without the requirement for ASC mediation (95). This is an unusual finding as no other study to date has shown inflammasome activation in the absence of ASC. Other studies implicate interaction of NOD2 with NALP1 leading to the formation of an inflammasome. This interaction leads to enhanced caspase-1 and NF-κB signalling. Another report has suggested an interaction between NOD2 and NALP3 (96), but this evidence has not been re-affirmed by subsequent studies (97). What also remains unclear is the action of the MDP PGN moiety, as it has been claimed to be a ligand for NALP1 (98) and NALP3 (99).

1.2.3 Peptidoglycan recognition proteins (PGRPs)

In addition to PGN moieties interacting with TLR2, NOD1 and NOD2, they can also be sensed by PGN recognition proteins, a class of PRRs that remain less well studied. Humans and mice express four secreted PGN recognition proteins (PGRPs) that bind directly to both Gram-positive and Gram-negative PGN (100). All PGRPs function in antimicrobial defence and can hydrolyse PGN, while most have diversified to carry out other host-defence functions. Insect and mammalian PGRPs defend host cells against infection through very different mechanisms. Mammalian PGRPs are directly bactericidal and can affect immune outcome. The potential role of this family of receptors in immunity to enteropathogen motifs was not investigated in the current study.

1.3 Gastrointestinal (GI) infections

GI infections affect over 1.7 billion people globally, particularly in the developing world. Diarrheal disease is the second leading cause of death in children under five years of age, as well as the leading cause of malnutrition. The development of vaccines has only been successful for a limited number of diarrhoea-causing agents therefore new therapeutic approaches are needed. But for this we need to understand host-pathogen interactions first. To date research has focused on *Salmonella,* interestingly *Salmonella* cases have been steadily declining over the last decade (EFSA), research effort is also shifting towards other bacteria. Two enteropathogens that have gained significance in the last two decades are *C. difficile* and *C. jejuni*. This is due to the increased incidence in cases of *Campylobacter* and *C. difficile* infections (see Section 1.1.2.2).

CDI is the leading identifiable cause of antibiotic-associated diarrhea. Remarkably, *C.difficile* is commensal in children and can often colonise them asymptomatically (101), but can cause disease in adults. In the last decade, the global incidence of CDI has increased dramatically due to the emergence and spread of epidemic strains associated with more severe disease, increased mortality, higher relapse rates and increased resistance to antibiotics (102). In contrast, *C. jejuni* is commensal in chicken and a main cause of gastroenteritis worldwide with young children being more susceptible to infection. *Campylobacter* infection is the leading cause of bacterial-mediated diarrhoeal disease, which is common in children under five (103). In Africa, a few studies have indicated that Campylobacteriosis is most common among children of young age (104). Among *Campylobacter* spp., *C. jejuni* is the most commonly isolated species from cases of gastroenteritis. Each of these two pathogens offers different challenges, given their different epidemiology, mode of infection and virulence factors. A better understanding of the host inflammation and immune mechanisms that

modulate the course of disease and control host susceptibility to these pathogens could lead to novel strategies for combating the challenges posed by these infections. This thesis addresses some fundamental host-pathogen interactions of *C. difficile* and *C. jejuni* with the GI tract.

1.3.1 *C. difficile*-host interactions

1.3.1.1 *C. difficile* taxonomy

C. difficile belongs to the *Clostridia* genus of the *Firmicutes* phylum, a group of large prokaryotes that encompass Gram-positive, rod-shaped, anaerobic bacteria. Clostridia are Gram-positive bacteria that form endospores and therefore have an ecological advantage for survival under adverse conditions. Members of the *Clostridia* class belong to one of three orders: *Clostridiales*, *Halanaerobiales* and *Thermoanerobacteriales* (105).

C. difficile is a member of the phylogenetic cluster XI of *Clostridia*, while other pathogenic species, such as *Clostridium perfrigens* and *Clostridium tetani* are members of *Clostridium cluster I*. It was initially identified in 1935 as part of the normal gut flora of neonates and named *Bacillus difficilis* due to its slow growth and the difficulty encountered in culturing the bacterium (106). In 1978, *C. difficile* was first identified as a pathogen and as a causative agent for antibiotic-associated diarrhea (107).

1.3.1.2 Commensal *Clostridia* species

The intestinal microbiome is composed of four major microbial phyla (*Firmicutes*, *Bacteroides*, *Proteobacteria* and *Actinobacteria*), divided into three distinct groups: *Bacteroides*, *Clostridium* cluster XIVa (also known as the *Clostridium coccoides* group) and *Clostridium* cluster IV (also known as the *Clostridium leptum* group) (108). Strains that fall within clusters IV, XIVa and XVIII of *Clostridia* lack prominent toxins and virulence factors. *Clostridia* make up roughly 60% of the total bacteria in the adult gut microbiome. They colonise the colon during the first month of life and populate the intestinal mucosa. This position brings them in close contact with IECs where they can act as effectors of physiologic, metabolic and immunologic events either on their own or via interactions with other resident microbes (16). In particular, commensal *Clostridia* play an important role in the metabolic welfare of colonocytes by releasing butyrate as an end-product of fermentation (109). A recent study showed that the relative abundance of *Clostridia* was much lower in infants suffering from necrotising enterocolitis (110). Interestingly, *Clostridia* species were used in a study to manipulate the microbiome for therapeutic purposes (111). Oral administration of a combination of *Clostridia* strains attenuated disease in mice models of colitis by enhancing Treg proliferation and inducing anti-inflammatory molecules such as IL-10. Through their metabolic role, *Clostridia* are emerging as key probiotic effectors in the intestine and a promising source of novel therapeutic strategies.

1.3.1.3 *C. difficile* associated diseases (CDAD)

1.3.1.3.1 Pathogenesis

We have focused on *C. difficile,* as it is currently, in clinical terms, the most important clostridial species. *C. difficile* is a major nosocomial bacterial pathogen that can cause a range of GI conditions in humans, these vary from simple diarrhoea to severe pseudomembranous colitis (PMC), an inflammation of the large intestine that leads to severe diarrhoea (112). *C. difficile* was first identified as the causative agent of PMC in 1977, thus defining its virulent capacity in susceptible individuals (113). In 2003, new hypervirulent strains appeared, first in Canada, and since then *C. difficile* infection (CDI) has spread globally (114). In the UK there are over 50,000 cases annually, with 7,000 deaths, effectively a person dying every hour in UK hospitals. CDI rates have also increased in groups that prior to 2000 were considered to be at low-risk, i.e. those with no immediate exposure to antibiotics, in children and in patients with IBD. CDI is considered a trigger in the aetiology of IBD and studies suggest that *C. difficile* gastroenteritis (115) may not only provide a trigger for flare-ups but may also be associated with disease severity in both adult and paediatric IBD (116),(117). A study demonstrated that patients with IBD who had associated *C. difficile* had a four times greater mortality and higher rates of colectomy than those without underlying IBD (118). Apart from humans, the bacterium can also infect other animal species, such as pigs, horses and dogs (119). The major virulence factors responsible for *C. difficile* pathogenesis are three toxins designated TcdA, TcdB and CDT (120),(121), where to date scientific endeavour, including work from our lab (122),(123), has focused on dissecting their function with the aim of generating antitoxin therapy (124). *C. difficile* remains less well-studied compared to other Gram-positive bacteria mainly due to the

difficulties in culturing anaerobes.

1.3.1.3.2 Asymptomatic colonisation

Although considered a major nosocomial pathogen, the majority of individuals who encounter *C. difficile* during their lifetime do not become ill. The bug will either transiently pass through the GI tract or exist asymptomatically in a low percentage (~ 4%) of healthy adults and up to 20% of hospitalised patients. Asymptomatic carriage is even more common in children (101), where *C. diffiicile* colonises 60 - 70% of newborns and infants up to 12-18 months of age when normal colonic microflora is established (125). *C. difficile* also colonises ~25% of children up to five years of age (126) in an asymptomatic fashion and this association, good or bad (102), has remained largely unexplored. This non-immunogenic cross-talk between *C. diffiicile* and the innate immune system has not been studied in detail. One study in a neonatal porcine model of CDI suggested that neonatal gut may not express the receptor for TcdA as an explanation for its disease-free association (127). However, this conclusion is unsatisfactory as *C. difficile* can secrete up to 3 toxins and specific lack of toxin receptor(s) in children has not been shown. One recent study indicates that *C. difficile* association in the neonate maybe asymptomatic but it is not silent, as the authors found an association between the presence of *C. difficile* and changes in the composition of the intestinal microbiota (128).

1.3.1.4 *C. difficile* cell wall

The Gram-positive cell envelope consists of a cytoplasmic membrane surrounded by a variety of glycan polymers and proteins. The cell wall, external to the membrane is a complex matrix of PGN and other polymers, including anionic polymers such as

teichoic acids and lipoteichoic acids polymers (129). The composition of the cell wall can vary significantly between species and is important in a variety of functions, such as host interaction, motility, invasion and microbe-microbe interactions.

1.3.1.4.1 Surface layer proteins

Many bacteria express a surface-exposed proteinaceous surface layer, termed the S-layer, which is formed by the self-assembly of monomeric proteins into a regularly spaced, two-dimensional array and is visible by electron microscopy. S-layers are found on both Gram-positive and Gram-negative bacteria and are some of the most abundant proteins in the cell; consisting of one or more (glyco)proteins, known as S-layer proteins (SLPs), that undergo self-assembly to form a regularly spaced array on the surface of the cell (130). *C. difficile* is unusual in expressing two S-layer proteins (SLPs), which are of varying size in a number of strains: the high-molecular weight (HMW) SLP and the low-molecular weight (LMW) SLP, both of which are produced by proteolytic cleavage of the precursor S-layer protein SlpA (131). This variation affects recognition by antibodies, which presumably reflects pressure from the host immune response (132). S-layer diversity in *C. difficile* is derived from a ~10 kb cassette that encodes SlpA, a protein translocase subunit (SecA2) and two cell wall proteins (CWPs), where recombinational switching occurs to generate antigenic diversity.

As the major surface antigen of *C. difficile*, the S-layer has the capacity to activate the immune system. The SlpA proteins (HMW and LMW SLPs) induce the release of IL-12, TNFα and IL10, but not the production of IL-1β from human monocytes and induce the maturation of human monocyte-derived DCs (133) Further work advanced these findings by showing that DC activation was dependent on Toll-like receptor 4 (TLR4). A

recent study showed that SLPs isolated from *C. difficile* induced the production of pro-inflammatory cytokines and chemokines in macrophages and increased macrophage migration and phagocytotic activity *in vitro*. Furthermore, treatment with SLPs led to the up-regulation of a number of cell surface markers important in pathogen recognition and antigen presentation (134).

1.3.1.4.2 Lipoproteins

Bacterial proteins of the Gram-positive or Gram-negative cell wall carry lipid modifications that facilitate anchorage. Lipoproteins of Gram-positive bacteria are processed by two key enzymes; the prolipoprotein diacylglyceryl transferase (Lgt) enzyme and the lipoprotein signal peptidase (Lsp) enzyme. The Lgt enzyme recognizes a so-called lipobox motif (LXXC) in the C-terminal region of the signal peptide of a premature lipoprotein and transfers a diacylglyceryl moiety to the cysteine residue of the lipobox. Subsequently, the Lsp enzyme cleaves the signal peptide resulting in a mature lipoprotein (Figure 1.3) (135). Deletion of the *lgt* gene is lethal to most Gram-negative bacteria (424). The acylation pattern depends on the existence of an N-acyl-transferase, which is mostly absent in Gram-positive bacteria and mycoplasma to result in the production of only diacylated LP. Gram-negative bacteria, on the other hand, synthesize mainly triacylated lipoproteins (136). Lipid modification of Gram-positive bacterial lipoproteins via Lgt has been described to be essential for innate immune activation (285, 320).

Lipoprotein coding genes comprise 2.25% of the *C. difficile* genome (137). A total of 85 lipoproteins have been identified through genomic sequencing, yet only very few have been isolated and studied in detail. Recently, the lipoprotein CD0873 was identified as

a putative adhesin and a CD0873 mutant showed significant reduction in adherence to IECs (138). Interestingly, immunization of mice with purified recombinant proteins from *C. perfingens* elicited protective immunity against challenge with the pathogen in a mouse model of infection (139). Lipoproteins have been shown to play a role in virulence in other bacteria, including *Enterococcus faecalis* and *Bacillus anthracis* (140).

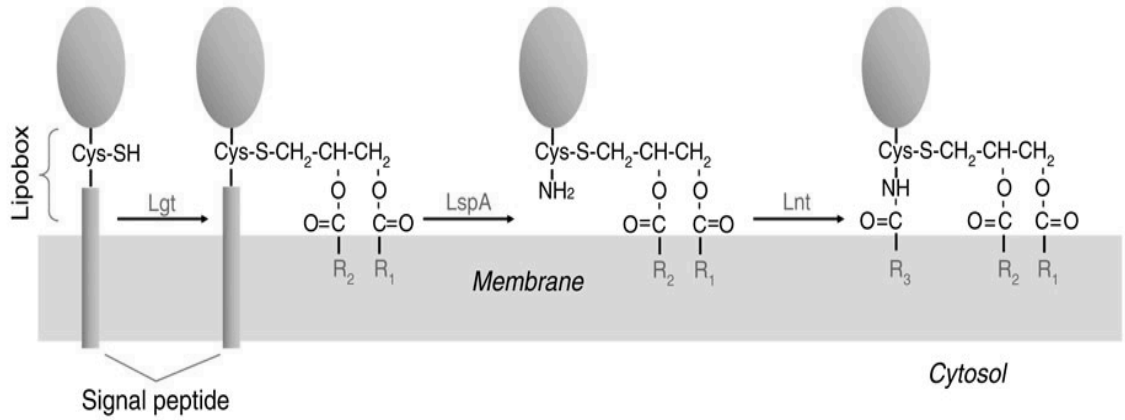

Figure 1.3. The peptide lipidation reaction.

(i) Diacylglyceryl modification of pre-prolipoproteins by phosphatidylglycerol pro*L*ipoprotein diacyl*G*lyceryl *T*ransferase (Lgt) to form diacylglyceryl–prolipoproteins; (ii) cleavage of signal peptide from diacylglyceryl-prolipoproteins by *L*ipoprotein *S*ignal *P*eptidase (LspA) to form apolipoproteins; and (iii) *N*-acylation of apolipoproteins by an apo*L*ipoproteiin *N*-acyl *T*ransferase (Lnt), resulting in mature lipoproteins. The third reaction only occurs in Gram-negative bacteria. [Adapted from ref. (424)]

1.3.1.4.3 Cell-wall glycopolymers

Most Gram-positive bacteria incorporate carbohydrate-based polymers termed cell-wall glycopolymers (CWG) into their cell envelopes (141). These are typically either membrane bound to glycolipids, termed lipoteichoic acids (LTA) (142), or linked to the PGN cell wall, termed wall teichoic acids (WTA) (143). The structure of CWGs varies between species but are typically polymers of sugar monomers containing phosphate groups such as 1,3-glycerol phosphate (Gro-P) or 1,5 D-ribitol-phosphate (Rbo-P) (144). CWGs differ according to their type of sugar, net charge and decoration of the repeating units.

C. difficile contains a rare, complex LTA polymer known as type V LTA, which has a proposed structure of α-D-GlcNAc(1–3)-α-D-GlcNAc repeating units linked through phosphodiester bridges. The second *N*-acetylglucosamine (GlcNAc) residue is further decorated with D–glyceric acid, and the polymer is retained in the membrane via a β-1-6-linked triglucosyl-diacylglycerol glycolipid anchor (145). Teichoic acids play a protective role, as a study found that d-alanylation of teichoic acids provides protection against antimicrobial peptides that may be essential for growth of *C. difficile* in the host. (146). The easy accessibility of CWGs in the cell wall makes these polymers interesting candidate targets for novel anti-infective strategies against emerging highly antibiotic-resistant bacteria such as *C. difficile,* particularly for new diagnostics and vaccines (129).

1.3.1.4.4 Peptidoglycan architecture

PGN is a major component of the Gram-positive bacterial cell wall, whereas it only makes up a thin layer in the periplasmic space of Gram-negative bacteria. PGN

surrounds the entire cell, contributing to the backbone framework for attachment of surface proteins and other polymers (143). It is crucial for plasma membrane integrity and confers resistance to antibiotics and anti-bacterial enzymes. The PGN biosynthesis pathway is targeted by a number of antibiotics, including penicillin and vancomycin. Gram-positive PGN is formed of long linear glycan strands cross-linked by short peptides (Figure 1.3). Some Gram-positive pathogens modify their PGN to resist host PGN-degrading lytic enzymes such as lysozymes. Lysozyme cleaves the (1-4) linkage between MurNAc and GlcNAc in the PGN backbone. Lysozyme is a critical host defence peptide found in abundance in macrophages and neutrophils (150). The two main mechanisms of PGN modification conferring lysozyme resistance involve acetylation of PGN glycan strands. These can be either N-deacetylation of the GlcNAc residues or O-acetylation of the OH group at C-6 of the MurNAc residues, (151).

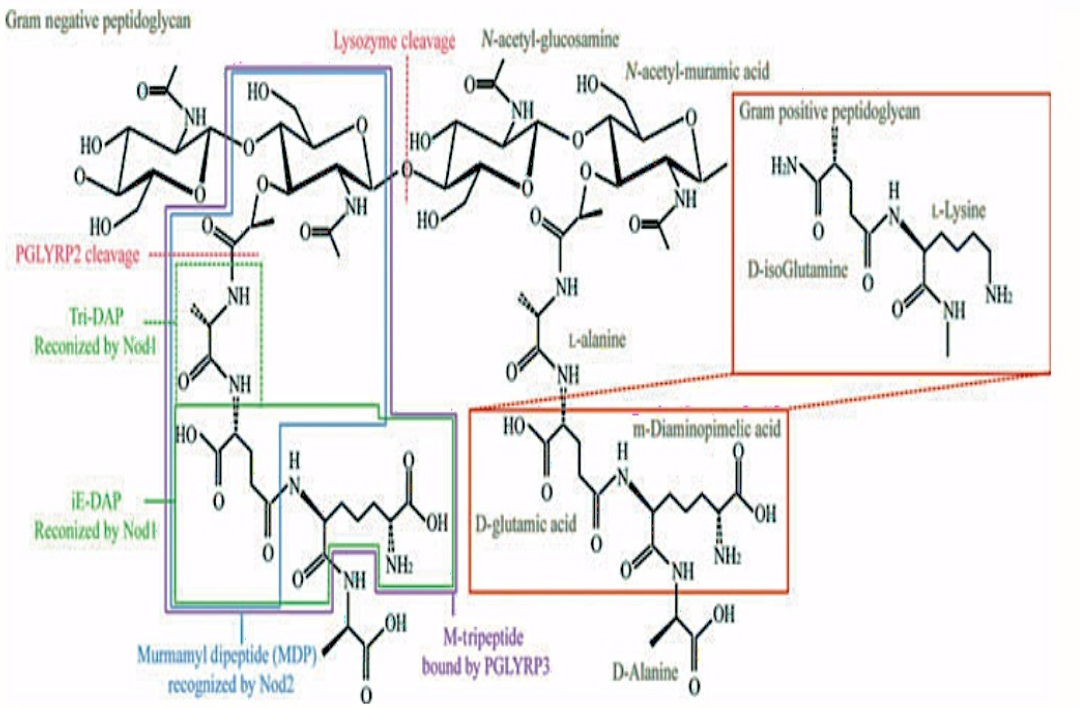

Figure 1.4. Peptidogylcan (PGN) structure and receptor binding motifs.

The chemical structure of DAP-type PGN, common in Gram-negative bacteria, is shown in black. The red inset shows the changes in structure common in Gram-positive bacteria, including the change of m-diaminopimelic acid (DAP) to L-Lysine, and D-glutamic acid to D-iso-glutamine. Pink: The cleavage sites of the amidase peptidoglycan recognition protein-2 (Pglyrp-2) and lysozyme are indicated with dashed lines. [Adapted from ref. (147)]

The genes responsible for these activities in Gram-positive bacteria are pgdA (PG N-deacetylase) and oatA (O-acetyltransferase), respectively. Both additional acetylation and deacetylation can confer increased lysozyme resistance depending on the microorganism. Increased lysozyme resistance is an important virulence factor for numerous Gram-positive bacteria, including *L monocytogenes* and *S. pneumoniae* which are N-deacetylated and *S. aureus* which is O-acetylated (152). Further to lysozyme resistance, the modification has been linked to bacterial survival and attenuation of host immunity. Inactivation of pgdA in *L. monocytogenes* revealed that the mutant was rapidly destroyed within macrophage vacuoles, followed by the release of its cell-wall components such as muropeptides and lipoteichoic acid (LTA). This led to the induction of a significant IFN-β response mediated by the PRRs TLR2 and NOD(153). Therefore, PGN N-deacetylation can function as a double-protection mechanism for *Listeria* against host innate defence by escaping the action of lysozyme and providing a mechanism to evade TRL2 and the NOD proteins.

1.3.1.4.5 *C. difficile* peptidoglycan

Until recently, little was known about the structure and biosynthesis of *C. difficile* PGN. Compared to other *Clostridium* species, such as *C. perfringens*, *C. difficile* exhibits lower susceptibility to β-lactams; i.e higher minimal inhibitory concentrations of β-lactams are needed to inhibit PGN biosynthesis (154). It also displays a preserved susceptibility to the antibiotic vancomycin despite the presence of a vanG-like operon that codes for a vancomycin-resistance gene (155). The fine structure of the vegetative cell wall of *C. difficile* was recently characterised by Peltier et al. and was found to have an original structure with many unique elements (156). The authors found a high percentage (93%) of GlcNAc in the *C. difficile* PGN to be deacetylated which accounted

for its resistance to lysozyme. Despite being intrinsically highly deacetylated, exposure to lysozyme can lead to additional deacetylation due to upregulation of a putative PGN deacetylase (157). The sequence of the peptide polymers mainly follows a pattern of L-Ala – L-Gln – A2pm – D-Ala, with an occasional addition of D-Ala as a 5th residue, or a substitution of D-Ala4 with a glycine residue. The same study found that 73% of *C. difficile* crosslinks involve L-D transpeptidation between A2pm3-A2pm3 of adjacent peptide polymers, with 27% of crosslinks involving D-Ala4-A2pm3. As discussed earlier, l,d-transpeptidases are insensitive to most β-lactams and contribute to *C. difficile* antibiotic resistance. As there is no pentaglycine cross-bridge *C. difficile* is not susceptible to the action of the antimicrobial agent lysostaphin (158). Furthermore, it is likely that any sortase substrates present will be covalently attached to the DAP cross-bridge as in *B. anthracis* (159). Beyond the structural role of PGN, this crucial moiety may also contribute to *C. difficile* asymptomatic survival and/or pathogenesis in genetically susceptible individuals.

1.3.1.5 Peptidoglycan (PGN) recognition

Given that the resident microbiota are normally non-invasive, NOD activation may be triggered without a cell invasion event. One hypothesis states that commensal bacteria come into contact with IECs or laminal propria DCs after stochastic diffusion through the mucus layer. Another suggestion is that free PGN fragments are released in the gut lumen and become endocytosed by IECs (160). In a study using radiolabelled PGN in mice, PGN fragments were detected in the serum suggesting that they had translocated through both the mucus layer and the intestinal barrier (161).

During their growth or cell division, bacteria are continuously shedding PGN muropeptides from the cell wall via the process of PGN turnover. In Gram-negative bacteria, turnover is coupled to recycling of the muropeptides back into the cytoplasm to be used in biosynthesis, yet only 30-60% of these muropeptides are recycled. Gram-positive organisms are not enclosed in a permeability barrier and the turnover products are released into the surrounding environment. Studies have validated the presence of NOD1 and NOD2 ligands in the supernatants of bacterial cultures from numerous strains (162). PGN turnover has not yet been studied in *C. difficile*, but in other species between 20% to 50% of total PGN is turned over in each generation (163). The process is catalysed by PGN hydrolases, enzymes that degrade bacterial muropeptides to generate smaller monomers or PGN fragments (164). So far, two PGN hydrolyses have been identified in *C. difficile*; the autolysin Acd (165) and the cell wall protein Cwp22 (132). Acd is an N-acetylglucosaminidase hydrolysing the bond between GlcNAc and MurNAc.

PGN can also be degraded by host hydrolytic enzymes known as hydrolyses. Hydrolases, such as lysozyme, are some of the most abundant antimicrobials present on the mucosal surface. The muramidase activity of lysozyme leads to hydrolysis of the β-1,4 glycosidic bond between the C-1 carbon of MurNAc and the C-4 carbon of GlcNAc residues of the PGN backbone (166). In addition, intraepithelial lymphocytes (IELs) express a PGN amidase, N-acetylmuramoyl-L-alanine amidase (also known as PGLYRP2), which can cleave PGN into large MDP-containing fragments (167). Finally, secreted pattern recognitions receptors for PGN -peptidoglycan recognition proteins (PGRPs) have been shown to bind and hydrolyse bacterial PGN (168). The plurality of bacterial and host peptidoglycan-degrading enzymes would suggest that PGN fragments recognised by innate immune cells are variable and may elicit diverse

responses. Identifying the structure and quantities of the NOD ligands being released is important for understanding how intestinal homeostasis is maintained.

1.3.1.5.1 Cytosolic peptidoglycan (PGN)

Although NODs have traditionally been seen as sensors of invading cytosolic bacteria, recent work suggests they are able to sense ligands released by extracellular pathogens. In the absence of direct cellular invasion by a pathogen, for example *C. difficile*, NOD signal transduction can take place by PGN entry to the cytosol; either by injection through bacterial secretion systems (169) or via delivery by outer membrane vesicles of Gram-negative bacteria (170),(171). Furthermore, two recent reports have found that epithelial cells and macrophages can take up PGN through endocytosis, demonstrating that NOD1 or NOD2 ligand uptake occurs through clathrin-mediated endocytosis (172),(173). Killing of internalised bacteria in the phagolysosomes is mediated by hydrolases amongst other factors, which are present in the granules of professional phagocytic cells (macrophages, neutrophils) and can cause bacterial cell lysis. Additionally, oligopeptide transporters, such as pH-sensing regulatory factor of peptide transporter-1 (PEPT1), can carry PGN fragments into the cytosol (173),(174). Finally, the transfer of PGN fragments can be mediated via gap junctions as demonstrated by the NOD1-dependent activation in cells located adjacent to infected cells (175). The presence of multiple mechanisms of PGN delivery suggests that NODs have a wider role in immunosurveilance of the gut microbiota than initially thought.

1.3.2 *Campylobacter jejuni*-host interactions

1.3.2.1 *Campylobacter jejuni*-mediated disease pathogenesis

Campylobacteriosis is the most frequent cause of food-borne gastroenteritis in the European Union with over 190,000 cases reported per year, although the actual number of cases is estimated at 9 million with the annual cost of infection estimated at 2.4 billion euros (European Food Safety Authority; EFSA). In the UK the rates of bacterial-associated gastroenteritis caused by *Campylobacter* sp. are as high as 9.3 cases per 1000 persons for community-acquired infections (176). Over 90% cases of human *Campylobacter* enteritis are caused by *Campylobacter jejuni*. Gastroenteritis is self-limiting in the majority of cases, lasting less than 1 week; although a small number of individuals develop post-infectious autoimmune pathologies. Despite the high incidence and economic burden presented by *C. jejuni*, its pathogenesis remains less well studied compared to other enteropathogens such as *Salmonella* typhimurium, *Shigella* sp., and *Escherichia coli*.

1.3.2.2 Taxonomy

C. jejuni belongs to the genus *Campylobacter*, order *Campylobacteriales*, which are members of the epsilon class of proteobacteria. It is a spiral, comma-shaped Gram-negative bacterium that utilises abipolar flagella for motility. *C. jejuni* was first described in 1931 in cattle suffering from spontaneous diarrhoea and termed *Vibrio jejuni* by Jones and colleagues (177). The *Campylobacter* genus was first identified as being separate from *Vibrio* in 1963 after Sebald and Véron noted the distinct biochemical properties of *Vibrio fetus* and *Vibrio bubulus* from other *Vibrio* species (178). In 1973, further analysis of microaerobic/anaerobic bacteria in the *Vibrio* taxa led to the re-classification of these bacteria into the *Campylobacter* taxon (179). Until 1973

Campylobacter sp. were primarily seen as veterinary pathogens, however in the 1970's the importance of *Campylobacter* sp. in human gastroenteritis became apparent as new techniques allowed the culturing of these micro-aerophillic bacteria from patients presenting with diarrhoea (180). There are currently 25 species of *Campylobacter* that have been isolated from a diverse array of hosts, both colonised without apparent symptoms and from those presenting with enteritis.

There are two subspecies of *C. jejuni*: *C. jejuni* subsp. *jejuni* (referred to as *C. jejuni*), which is the major cause of human *Campylobacter* enteritis (campylobacteriosis), and *C. jejuni* subsp. *doylei*. Subsp. *doylei* is the cause of very few cases of gastroenteritis in comparison to subsp. *jejuni* and the two subspecies differ considerably in distribution and incidence. The second most common cause of campylobacteriosis is *C. coli*, which is genetically the closest relative of *C. jejuni* and accounts for a small number of cases of human disease (181). While over the past decade the importance of other enteropathogens in human disease has become increasingly appreciated, the fastidious growth conditions of these *Campylobacter* sp. has resulted in underrepresentation of these organisms in clinical diagnosis (182).

1.3.2.3 Clinical manifestations

Acute gastroenteritis is the major clinical manifestation in humans infected with *C. jejuni*. Clinical symptoms include watery or bloody diarrhoea, often accompanied by abdominal cramps and fever (183). Incubation times of between 24-72h have been reported and symptoms often resolve within one week although they may persist for longer. In the majority of cases in the developed world, campylobacteriosis induces inflammatory diarrhoea, with polymorphonuclear (PMN) cells and erythrocytes present

in the stools (184). Symptoms are generally self-limiting, although immunocompromised patients, such as HIV patients and the elderly, are more likely to have extended gastroenteritis and develop bacteremia (185) which suggests host immunity plays a major role in limiting systemic spread.

Clinical manifestations of *C. jejuni* infection differ in people from developing countries who, mainly due to their more frequent exposure to multiple *C. jejuni* strains, develop fewer symptoms (183). The protective immunity acquired by repeated exposure protects only from symptomatic disease but not colonisation (186). Gastroenteritis is more commonly seen in children and it is rare for adults to develop symptomatic disease which suggests that exposure to multiple strains in early childhood contributes to the development of protective immunity. On the other hand, visitors from countries with no pre-existing *C. jejuni* immunity develop the more serious symptoms of bloody diarrhoea when infected in developing countries (187). Finally, although no differences have been found in strains isolated from symptomatic and asymptomatic infection (188), full genome sequencing of *C. jejuni* may reveal new associations.

1.3.2.3.1 Inflammatory Bowel Disease (IBD)

Increased risk of developing IBD has been associated with both *Campylobacter* and *Salmonella* infection (189). However, the evidence remains contradictory with a more recent study demonstrating that increased sampling bias around the onset of IBD is responsible for the increased detection rates of *Campylobacter* and *Salmonella* in newly diagnosed IBD patients (190).

1.3.2.3.2 Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is an autoimmune neuropathy resulting from the production of autoantibodies that cause de-myelination of nerve gangliosides in the peripheral nervous system. Symptoms often begin in the lower extremities, and can ascend through the body resulting in full neuromuscular paralysis, with one third of patients requiring ventilator-assisted breathing. The majority of GBS cases are preceded by gastroenteritis, and the discovery of molecular mimics between *C. jejuni* lipooligosaccharide (LOS) and nerve gangliosides led to the identification of *C. jejuni* infection as a major aetiological agent of acute motor axonal neuropathy (AMAN), a variant of GBS. Host factors are also important as GBS-associated *C. jejuni* strains have been isolated from patients suffering from uncomplicated enteritis (192).

1.3.2.4 *C. jejuni* surface structures

Like many other mucosal pathogens, the surface structures of *C. jejuni* are heavily glycosylated with approximately 8% of its genome encoding proteins implicated in surface carbohydrate structures (Figure 1.4) (193). *C. jejuni* encodes both N-linked and O-linked glycosylation pathways for the modification of surface and flagella proteins respectively. Genome sequencing of *C. jejuni* identified the pathogen's ability to promote phase-variation of many genes involved in glycosylation (193). Long stretches of repeated C or G bases called "homopolymerlc tracts" increase slip-strand mispairing which can result in genes being "switched on" or "switched off". The importance of these glycosylation systems in bacterial-driven pathogenesis is only now being appreciated.

1.3.2.4.1 Lipooligosaccharide

Lipopolysaccharide/lipooligosaccharide (LPS/LOS) forms an integral part of the outer membrane of Gram-negative bacteria. *C. jejuni* expresses LOS, which consists of a hydrophobic lipid A anchored to the outer membrane and an extracellular carbohydrate oligosaccharide (OS) that lacks the repeating carbohydrate O-antigen of LPS molecules. The lipid A anchor is essential for membrane integrity and complete mutation of *C. jejuni* LOS is lethal (194). Variation in OS length and composition is a key strategy employed by Gram-negative bacteria that allows them to evade and modulate host immunity.

The sugar components of the disaccharide lipid A backbone can be 2,3-diamino-2,3-dideoxy-D-glucose (GIcN3N) or a D-glucosamine (GlcN) moiety (196). The lipid A backbone is hexacylated with either palmitic (14 carbon) or myristic (16 carbon) acid (196). GIcN3N contains two amide-linked palmitic/myristic acid chains, in comparison GlcN contains one amide and one ester linked fatty acid chain. The two additional palmitic acid chains are attached to the palmitic and myristic acid of the sugar proximal to the OS, making the disaccharide asymmetrical.

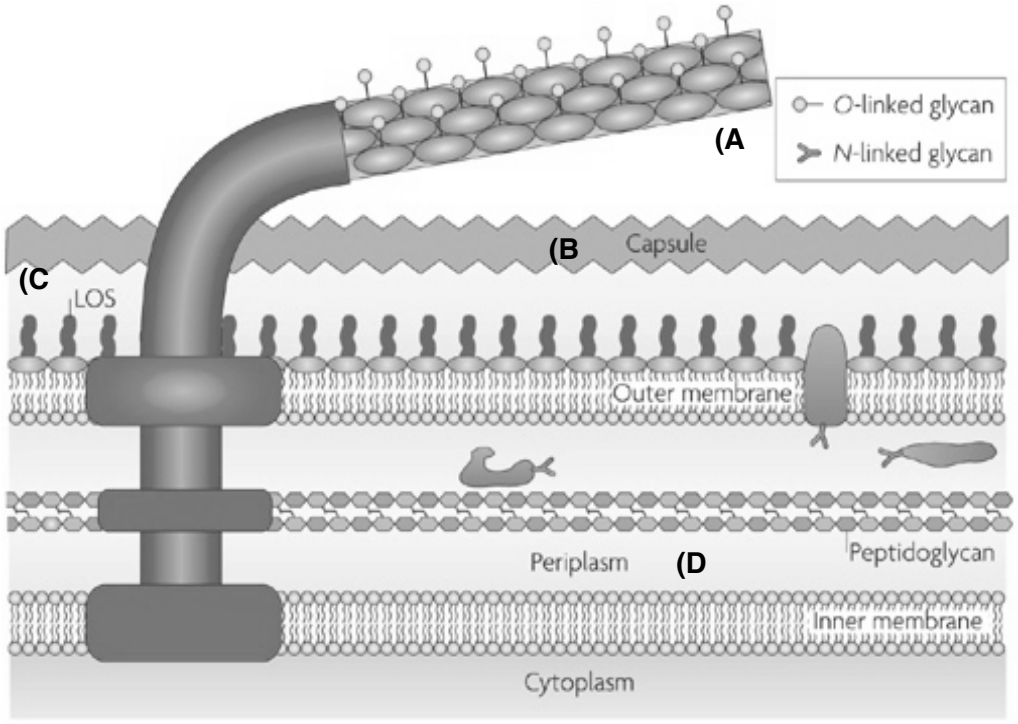

Figure 1.5. C. jejuni surface structure features.

(a) The flagellum is anchored into both the inner and outer membrane. Flagellin proteins, FlaA and FlaB are O-linked glycosylated with sialic acid-like structures. (b) The capsule is a highly branched polysaccharide structure. (c) Lipooligosaccharide (LOS) is an essential feature of the outer membrane (d) Periplasmic and outer membrane embedded proteins are modified by the N-linked glycosylation system. [Adapted from ref. (195)].

The most common backbone composition is GlcN3N-GlcN, although both GlcN3N-GlcN3N and GlcN-GlcN structures have been detected (197). GlcN3N sugars reduce both the ability of *C. jejuni* LOS to activate Toll-like receptor 4 (TLR4) and the sensitivity of live *C. jejuni* to cationic AMPs (198). The disaccharide backbone can be modified with the addition of both phosphate (P) and O-phosphorylethanolamine (PEA) on either or both sugars (196),(197). The gene responsible for the addition of PEA groups onto the lipid A, Cj02S6, is also responsible for the modification of the flagellar rod protein, FlgG, with PEA (199). The phosphorylation pattern and disaccharide composition of *C. jejuni* varies between strains and is also dependent on the growth phase (197).

The *C. jejuni* OS consists of a mainly conserved inner core OS proximal to the lipid A backbone, and an outer core OS which varies significantly between strains suggesting the two OS cores are under differential selective pressure (Figure 1.5). The inner core OS contains two 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) residues, two heptose (Hep) residues, with two branched glucose (Glu) residues on each heptose, and one PEA/P residue on the heptose proximal to the KDO (200). The outer core OS of 11168 and other *C. jejuni* strains are composed of galactose (Gal), glucose, N-acetyl-D-galactosamine (GalNAc), and N-acetylneuraminic acid (Neu5Ac; sialic acid) residues. The number of each sugar varies between strains and some strains lack sialic acid and GalNAc residues completely. The LOS biosynthesis locus in the *C. jejuni* genome is hypervariable and is composed of between 10 - 20 genes (201). To date 19 different LOS classes have been identified with varying levels of homology (202),(201). Classes A, B, C, M and R are responsible for the sialylation of LOS and sequencing has revealed minor genetic alterations within strains of the same LOS class as an additional mechanism which *C. jejuni* employs to vary the outer core OS (201). Strains

Figure 1.6. Structure of the core oligosaccharide of NCTC 11168 LOS.

The carbohydrate structures analogous to the gangliosides GM1a and GM2 are bracketed. Kdo, 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid; Hep, **L**-*glycero*-**D**-*manno*heptose; Glc, glucose; Gal, galactose; Neu5Ac, *N*-acetylneuraminic acid or sialic acid; GalNAc, *N*-acetyl-D-galactosamine.

isolated from livestock show a greater propensity for LOS sialylation compared to non-livestock sources. Interestingly, the number of sialic acids correlated with TLR4 activation and release of TNFα in human monocytes (203), suggesting that LOS variation is critical in monocyte activation.

The high degree of variability in the outer core OS structure suggests that it may be under differential selective pressure compared to the inner core. Indeed many structural features of the outer core OS are critical in host-interactions (204),(205),(206). An important consequence is the molecular mimicry observed between the OS outer core structure and host gangliosides and other glycans (207),(208). In addition to gangliosides, *C. jejuni* LOS can share structural features with P-blood group, paragloboside, lacto-N-biose and sialyl-lewis-c units (208). Presumably, mimicry of host glycans is a potential immune evasion strategy, as adaptive immune cells with specificities for these glycans will be deleted leading to increased peripheral tolerance.

1.3.2.4.2 Flagella

C. jejuni possess a single flagellum at either pole; a structure critical for motility as well as colonisation of their avian host (209). The flagellar filament is composed of proteins FlaA and FlaB. FIaA is a major structural component since mutation of the flaA gene results in a non-motile phenotype and severe truncation of the flagellum (210). On the other hand, the ΔflaB mutant bears full-length flagella and exhibits only partially impaired motility. The flagellar filament is embedded into both the inner and outer membrane via a basal body and hook complex composed of multiple structural proteins, which allow free rotation of the filament to facilitate motility. The flagellar filament of *C. jejuni* is a coiled-coiled structure composed of 7 protofilaments with a central lumen (211). Unlike many other well-known Gram-negative enteropathogens

(e.g. *Salmonella*, *Yersinia* sp.), *C. jejuni* lack a functional secretion apparatus (such as type III), instead *C. jejuni* secretes effector proteins, invasion antigens (Cia proteins) and FlaC through the flagella apparatus (212). This process requires the basal body and hook structure, and one of the FlaA or FlaB proto-filaments but not both.

Flagellin proteins are O-linked glycosylated with sialic acid (SA) like structures making up nearly 10% of its total mass. O-linked glycosylation involves the addition of a sugar moiety to the hydroxyl group of a serine or threonine residue of a target protein although no target amino acid sequence has been identified. O-linked glycosylation of flagellin proteins is considered to be critical for the assembly of functional flagella (213). Rather than SA, *C. jejuni* flagellin contains pseudaminic acid (Pse) and legionaminic acid (Leg), both derivatives of SA like structures encoded by the Pse and Ptm biosynthesis pathways respectively (214),(215),(193). All *C. jejuni* strains studied to date express either Pse and its derivatives (e.g. strain 81-176) or derivatives of both Pse and Leg (e.g. strain 11168). In strain 11168 the flagellin glycosylation genetic cluster is composed of 50 genes, which encode Pse and Leg pathways (193). In contrast, strain 81-176 contains only the Pse synthesis pathway. Exposure of glycosylated residues on the external surfaces of the flagellum suggests that these modifications may play a role in interaction with the host (216). In fact, mutation of the Leg structures in strain 11168 dramatically decreases the ability of *C. jejuni* to colonise the chicken GI tract (213). As an immune evasion strategy, *C. jejuni* and other proteobacteria have evolved mutations in their flagella such that it no longer interacts with TLR5 (217). Instead, *C. jejuni* flagella activate the immunoinhibitory Siglec-10 receptor via Pse structures on its surface to induce production of IL-10 (218). Therefore, flagella may play an important role in the interaction of *C. jejuni* with host immunity by contributing to bacterial survival.

1.3.2.4.3 Capsule

The capsule consists of a high molecular weight polysaccharide component originally thought to be LPS but later identified as capsular polysaccharide (CPS) based on the genome sequence (219),(220). The CPS and not the LOS was also found to be the determinant in the heat-stable Penner serotyping scheme. Similar to the LOS, phase variable cps genes have been also been identified, implying that CPS may interact with the host. The genetic locus for *C. jejuni* 11168 CPS contains 38 genes. Those involved in CPS polymerisation and translocation are conserved between strains however the internal region encoding the CPS repeating units is highly variable in size. Indeed, CPS was found to play a role in dampening the pro-inflammatory cytokine responses in DCs as well as invasion of IECs (221),(222).

1.3.2.4.4 N-linked glycosylated proteins

N-linked glycosylation involves the attachment of a sugar moiety to the amide nitrogen of an asparagine residue. *C. jejuni* was the first microorganism found to employ an N-linked glycosylation system (223). N-linked glycosylated proteins are highly conserved between strains (224), unlike other *C. jejuni* surface structures. A heptasaccharide composed of a single bacillosamine moiety attached to a linear chain of 5 GalNAc residues with one tertiary glucose residue forms the structure of all *C. jejuni* N-linked carbohydrates. N-linked glycosylation is thought to play a role in colonisation of the chicken gut, adherence and invasion of human IECs and modulation of DC immunity (225),(222),(226).

1.3.2.5 Innate Immunity to *C. jejuni*

Understanding of *C. jejuni* pathogenesis lags behind that of other enteric pathogens in part due to the lack of a good small animal disease model. Chick models are used for *C. jejuni* colonisation studies however the pathology associated with human disease is not observed. Wild-type (WT) mice of multiple genetic backgrounds show limited colonisation of *C. jejuni* and pathology is even more infrequent (227),(228). Until recently, mice with genetically altered immune systems such as IL-10 knock-outs or mice depleted of lymphocytes were the most frequently used animal model systems (227),(71),(228). In 2011, a model replacing the enteric flora of WT mice with human flora has been described (229). These mice were colonised with *C. jejuni* and subsequently developed inflammatory responses. Larger animal models, such as new-borne piglets, are also used for pathogenesis studies however, high cost and limited genetic tools make them less desirable (230).

1.3.2.5.1 Dendritic cells

Human peripheral blood monocyte-derived DCs (mDCs) or bone marrow-derived DCs (BMDCs) are frequently used in *in vitro* co-culture assays. Both DC types model a more inflammatory mDC subset, which is more likely to be present during intestinal infection and inflammation. *C. jejuni* is readily phagocytosed and killed by human mDCs and BMDCs (231),(228). Other enteropathogens such as *Salmonella enterica* are able to survive within DCs which may account for the greater incidence of systemic disease (232) compared to campylobacteriosis. *H. pylori*, which is highly related to *C. jejuni,* can also replicate within BMDCs for a limited time (233).

1.3.2.5.2 Macrophages

Macrophage-mediated immunity to pathogens such as *C. jejuni* is central to the outcome of infection. Studies frequently utilise the pre-monocytic THP-1 cell line which assumes a macrophage-like phenotype upon stimulation with phorbol-12-myristate 13-acetate (PMA) (234). Unlike resident intestinal macrophages, TLR ligands elicit a pro-inflammatory response in PMA-differentiated THP-1 cells (dTHP-1 cells). *C. jejuni* bacterial cell lysates induce caspase-dependent IL-1β secretion from dTHP-1 cells (235), as well as apoptosis via a caspase-independent pathway. According to some studies, *C. jejuni* can survive within monocytes/macrophages for up to 7 days, whereas it is readily killed by murine BM macrophages and dTHP-1 cells (236),(237),(218). This discrepancy may be attributed to the use of distinct cell types although it is not currently known how resident macrophages respond to *C. jejuni*.

1.3.2.5.3 Neutrophils

Neutrophils along with eosinophils, basophils, and mast cells make up the polymorphonuclear (PMN) cells or granulocytes. Neutrophils engulf and rapidly kill bacteria via the production of reactive oxygen species (ROS) in the phagolysosome. Neutrophils have the most potent phagocytic and bactericidal capability of all the phagocytes and are therefore central in the host response to infection. PMNs have been implicated in *C. jejuni*-mediated inflammatory pathology as their degree of infiltration of the GI mucosa correlates with the degree of inflammation (238). Neutrophil chemoattactants such as IL-8 are secreted by IECs in response to *C. jejuni* (239),(240). In general, neutrophils act to limit the systemic spread of *C. jejuni* although

as a result they contribute toxic ROS (241). Interestingly, GI colonisation in chicken is not associated with recruitment of avian neutrophils (heterophils) (242).

1.3.2.5.4 Cytokine responses to *C. jejuni* infection

C. jejuni-mediated infection induces a pro-inflammatory response from IECs as well as innate and adaptive immune cells. Chemokines such as IL-8, and monocyte chemoattractants CCL2 and CCL4 are secreted from *C. jejuni*-stimulated IECs (244),(237). *Ex vivo* co-culture experiments with human intestinal biopsies also show elevated levels of IL-8 (245). *C. jejuni* also stimulates pro-inflammatory cytokine induction in DCs and macrophages (231),(228). Increase in acute-phase response inducers TNFα and IL-6, as well as Th1 and Th17 polarising cytokines IL-12 and IL-23 respectively has also been noted (246). Similar to IEC responses, no dramatic difference between human and chicken *C. jejuni*-stimulated macrophages/monocytes has been observed (247). Minimal levels of IL-1β have also been detected in *ex vivo* stimulated biopsies (246). Initial reports suggesting induction of the potent anti-inflammatory cytokine IL-10 is flagellin-dependent were later confirmed by a study showing the involvement of Pse structures of the *C. jejuni* flagella in IL-10 induction (222),(218).

1.3.2.6 Immune recognition of *C. jejuni*

1.3.2.6.1 Toll-like receptors

C. jejuni activates human TLR1/2/6 and TLR4/MD2 receptors (247). *C jejuni* LOS is the ligand for TLR4/MD-2 (248) while di- and tri-acylated lipoproteins are predicted to bind TLR1/2 and TLR2/6. *C. jejuni* can successfully colonise MyD88-/- but not WT mice highlighting the role of TLR signalling in colonisation resistance (249). In chicken, *C. jejuni* is able to stimulate TLR2 and TLR4 homologs as well as the chicken TLR9 ortholog, chTLR21 (247). However, activated chicken TLR4 is unable to induce type 1 IFN response unlike human TLR4. Whether these discrepancies play a role in the different outcomes of *C. jejuni* infection in humans and chicken requires clarification.

1.3.2.6.2 NOD receptors

In IECs, *C. jejuni* activates NOD1 but not NOD2 to induce the secretion of human β-defensin 2 (HBD2) and IL-6 (78) NOD1 activation also minimises intracellular *C. jejuni* numbers. *C. jejuni* can also activate NOD2 in a reporter cell line although the functional consequences of this remain unknown (78),(250).

1.3.2.6.3 Glycan receptors

Bacteria employ glycosylation of their surface structures to gain potential fitness advantages. These include survival in harsh and varying environments, assembly of

surface structures, adherence and invasion of target cells and subversion of host immunity (251). In contrast to protein, glycans are less immunogenic as they are not generally presented on MHC molecules to T cells, and are therefore mainly T-cell independent antigens. There is mounting evidence of the capacity of polysaccharides from commensal bacteria to influence the mucosal immune system. Polysaccharide A (PSA), a component of the CPS of the human commensal *B. fragilis*, activates TLR2 on mucosal CD4' T cells inducing Treg differentiation by inducing IL-10, TGF-β and Foxp3 and further immunoinhibitory signalling (252). Bacterial glycan structures can bind to a wide variety of host receptors expressed on immune and non-immune cells. With respect to *C. jejuni*, these interactions have been shown to both enhance and dampen pro-inflammatory immune responses (226,248).

1.3.2.6.4 Sialic-acid binding glycan receptors

Sialic-acid binding immunoglobulin-like receptors (Siglecs) are a group of receptors largely expressed on the surface of haematopoietic cells. Siglecs are composed of a single trans-membrane domain, multiple extracellular Ig domains that bind ligand, and a cytoplasmic intracellular inhibitory motif (ITIM) domain which exerts regulatory function (253). Some Siglecs contain an intracellular growth factor receptor binding protein-2 (Grb2) binding motif and ITIM-like motifs that do not conform to canonical ITIM sequences, however the functional consequences of these remains largely uncharacterised (254). There are two sub-categories of Siglec receptors based on sequence similarity. The first includes Sialoadhesin, CD22, Siglec-4 and -15; the second subset share sequence homology with CD33 and are termed the CD33-related Siglecs(253),(255). The CD33-related receptors are rapidly evolving, with variation in the numbers of receptors between species, even in higher order mammals. There are

currently 10 known human CD33-related Siglecs which are expressed in both innate and adaptive immune cells (256). Although humans display the most rapid rate of Siglec evolution, lower Siglec expression in human compared to chimpanzee T cells has been implicated in the heightened reactivity of human T cells to many stimuli (257). Additionally, lower expression is thought to contribute to the "over-reactivity" of the human immune system leading to diseases such as asthma and rheumatoid arthritis.

Siglecs bind sialylated structures (most often sialylated glycans) via the terminal V-set Ig domain, and have varying specificities for the linkage of the sialic acid and the underlying glycan structure, which can be present on both host cells and microbes. Interestingly, recent reports have also highlighted the ability of Siglecs to bind to both host and pathogen non-sialylated ligands. Siglec-5 can bind the β-protein of group B *Streptococcus* (258), and Siglec-10 can bind vascular adhesion protein-1(259). However it is unlikely that these interactions occur via the sialic-acid binding pocket.

Siglec engagement has been implicated in many cellular processes including endocytosis, apoptosis, cellular activation, and proliferation(253). The immunomodulatory capability of Siglecs is often via the modulation of TLR signalling (260) (Figure 1.6). Siglecs can bind to host cell surface sialic acid both via cis (on the same cell) and trans (different cell) interactions. Binding of Siglec-10 (murine Siglec-G) by the receptor CD24 leads to down-regulation of NF-κB signalling, protecting mice in a inducible liver necrosis model (261). This suggests that Siglecs play a role in limiting immune responses to DAMPs. This immune modulatory capability of Siglecs may have been exploited by pathogens during evolution since binding to these receptors potentially reduces immune activation and therefore increase chances of establishing infection. Many sialylated pathogens engage Siglecs, including *C. jejuni*(262),(263). Engagement of Siglec-9 by sialylated CPS and Siglec-5 by β-protein by Group B

Streptococcus reduces the oxidative burst of neutrophils and increases intracellular survival (264),(265). Engagement of murine Siglec-E by *Trypanosome cruzi* reduces DC IL-12 production which alters subsequent T-cell activation (266). *C. jejuni* strains that contain terminal α2,8-linked and α2,3-linked sialic-acid on their LOS bind to Siglec-7 and sialoadhesin respectively (262),(263). The differential expression of these linkages drives alternative T cell responses, a factor which is hypothesised to impact on the development of GBS post *C. jejuni* infection (267).

Multiple hypotheses have been proposed for the rapid evolution of CD33-related Siglecs. One hypothesis is the need to keep pace with the changing host slalome (253). As a high percentage of sialic-acid containing pathogens infect humans; this raises a second hypothesis that rapid evolution might allow Siglecs to act as potential PRRs aiding microbial detection. However, the evolution of paired Siglec receptors which share structural identity of the ligand binding domain - but lack the intracellular signalling domain – is thought to be a decoy strategy by the host to counteract the immune inhibition pathogens achieve on Siglec engagement, therefore supporting the first hypothesis (255). Their exact role in microbe recognition and immune evasion remains open.

1.3.2.6.5 C-type lectin receptors (CLRs)

Similar to Siglec receptors, CLRs are a group of receptors predominantly found on the surface of immune cells. CLRs contain a single trans-membrane domain, a C-type lectin-like domain involved in ligand binding, and often an intracellular signalling domain (268). Similar to Siglecs, engagement of CLRs often leads to dampening of TLR signalling, and has therefore been described as an immune evasion strategy by

certain pathogens. *Mycobacterium tuberculosis* and *Candida albicans* are able to induce IL-10 secretion by engaging the CLR DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), via prolonged NF-κB activation (269). *C. jejuni* can bind the CLR macrophage-binding galectin (MGL) through GalNAc residues on N-linked glycosylated surface proteins. Additionally, the LOS from certain strains bearing a terminal GalNAc residue can also bind MGL. The interaction with MGL was shown to reduce IL-6, indicating that certain *C. jejuni* surface structures may actively promote immune suppression (226). Furthermore, *H. pylori* interaction with DC-SIGN reduces cytokine induction which stunts Th1 responses affecting T-cell polarization (270).

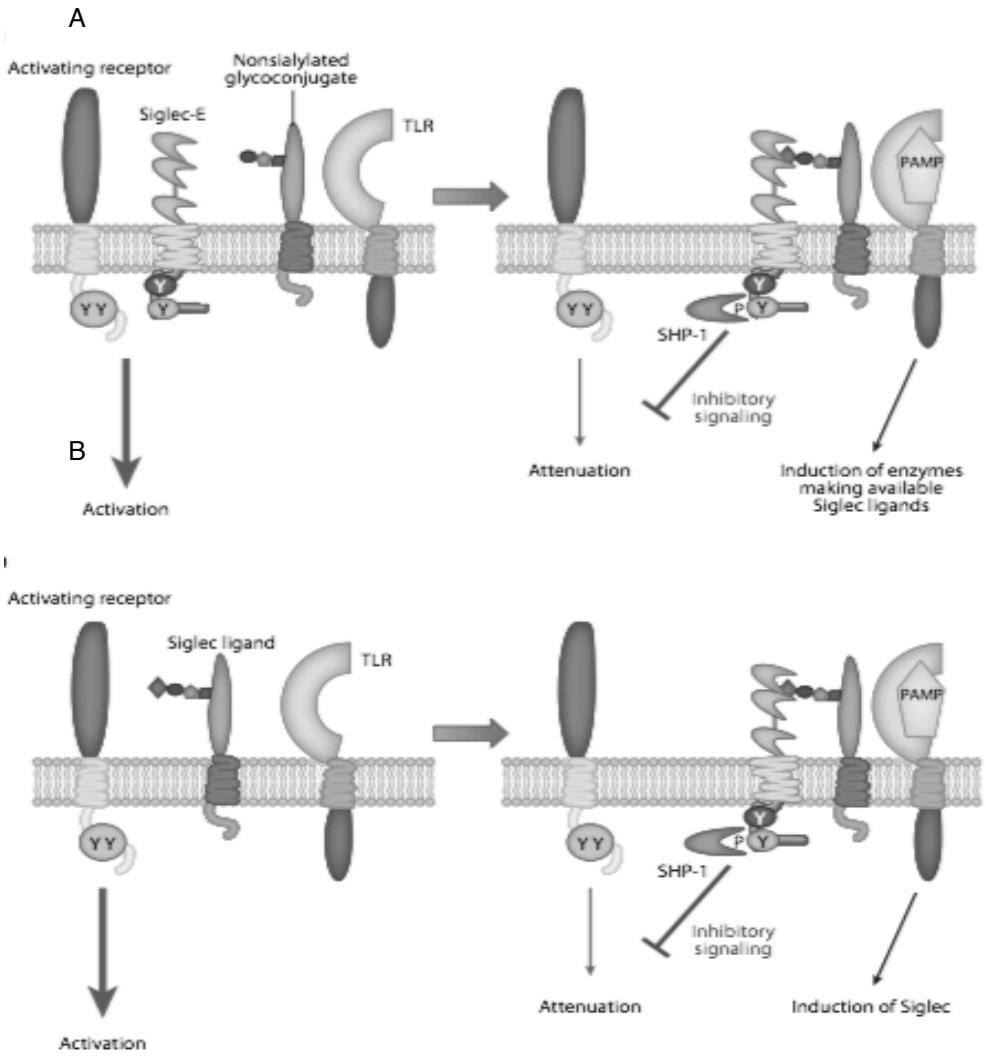

Figure 1.7. Siglecs in the regulation of inhibitory signalling.

(A) Activation of a TLR by a PAMP results in the generation of Siglec ligands through activation of phagocytic mechanisms that regulate ligand availability. The generation of a Siglec ligand results in inhibitory signalling being brought into play. (B) Activation of a TLR by a PAMP results in the induction of Siglec expression, which in turn brings inhibitory signalling into play. [Adapted from ref (398)].

1.3.2.7 Immune Signalling

1.3.2.7.1 Nuclear factor Kappa-Light-Chain Enhancer of Activated B Cells

In vitro inhibitor studies have revealed a critical role for NF-κB signalling in the induction of inflammatory cytokines both in *C. jejuni*-stimulated IECs and DCs (71),(218). In a gnobiotic IL-I0-/- NF-κB reporter murine model, pathology was associated with NF-κB activation in LP mononuclear cells and induction of IL-12p40 and TNFα (71). However, the use of NF-κB inhibitors did not ameliorate *C. jejuni*-mediated pathology suggesting this signalling pathway alone is not responsible for the inflammatory nature of campylobacteriosis in this model.

1.3.2.7.2 Mitogen-activated protein kinases (MAPKs)

MAPKs are a family of serine/threonine kinases with multifaceted roles in cellular biology, including cellular proliferation and survival, transcriptional control, cellular movement, as well as induction of inflammatory responses after microbe recognition. MAPKs are activated by upstream MAPK kinases, which in turn become activated by MAPK kinase kinases (MAP3K) (Figure 1.7). Once activated MAPKs regulate a number of different transcription factors involved in immune function. Regulation of activation is tightly controlled, for example MAPKs can regulate their own activation by negative feedback mechanisms involving activation of one of three classes of protein phosphatases (271).

The MAPK cascade is composed of three major groups of kinases: extracellular signal-regulated kinases (ERKs) comprising ERK1 and ERK2; JuN N-terminal kinases (JNKs)

comprising JNK1 and JNK2; and p38 (272). Each group exerts cell-specific effects on various cellular processes. p38 and JNK are linked to pro-inflammatory responses. p38 was first identified as the target of pyridinyl imidazole, a group of compounds capable of inhibiting monocyte TNFα and IL-1 secretion (273). ERK has been linked to pro-inflammatory responses in DCs (274) but it can also influence IL-10 and TGF-β expression, and the development of T-cell immunity (275). MAPKs activate transcription factors that differentially regulate inflammatory gene expression. For example TLR-mediated ERK-dependent activation of AP-1 is essential for IL-23 p19 gene expression, although this pathway does not elevate IL-12/IL-23 p40 expression (276).

The profile of transcription factor activation by these pathways tailors a specific immune response. TAK1 is an important MAP3K, downstream of many PRRs including TLRs, and is capable of activating all three MAPK members. In addition, TAK1 can activate NF-κB directly through the ubiquitination and degradation of inhibitory IKK molecules (277). ERK and p38 signalling consolidates in the activation of mitogen and stress activated protein kinase (MSK) 1 and 2 which in turn activate transcription factors that bind the IL-10 promoter. The transcription factors cAMP-responsive-element-binding protein (CREB) and activating transcription factor 1 (ATF1) become activated by MSKs in LPS-stimulated mouse macrophages (278). In addition to gene regulation, MAPKs can also participate in microbe uptake. Syk is a tyrosine kinase downstream of many CLRs and is involved in multiple immune functions including cytoskeletal rearrangement for phagocytosis. ERK-mediated Syk activation has been linked to the phagocytosis of *Francisella tularensis* by macrophages (279).

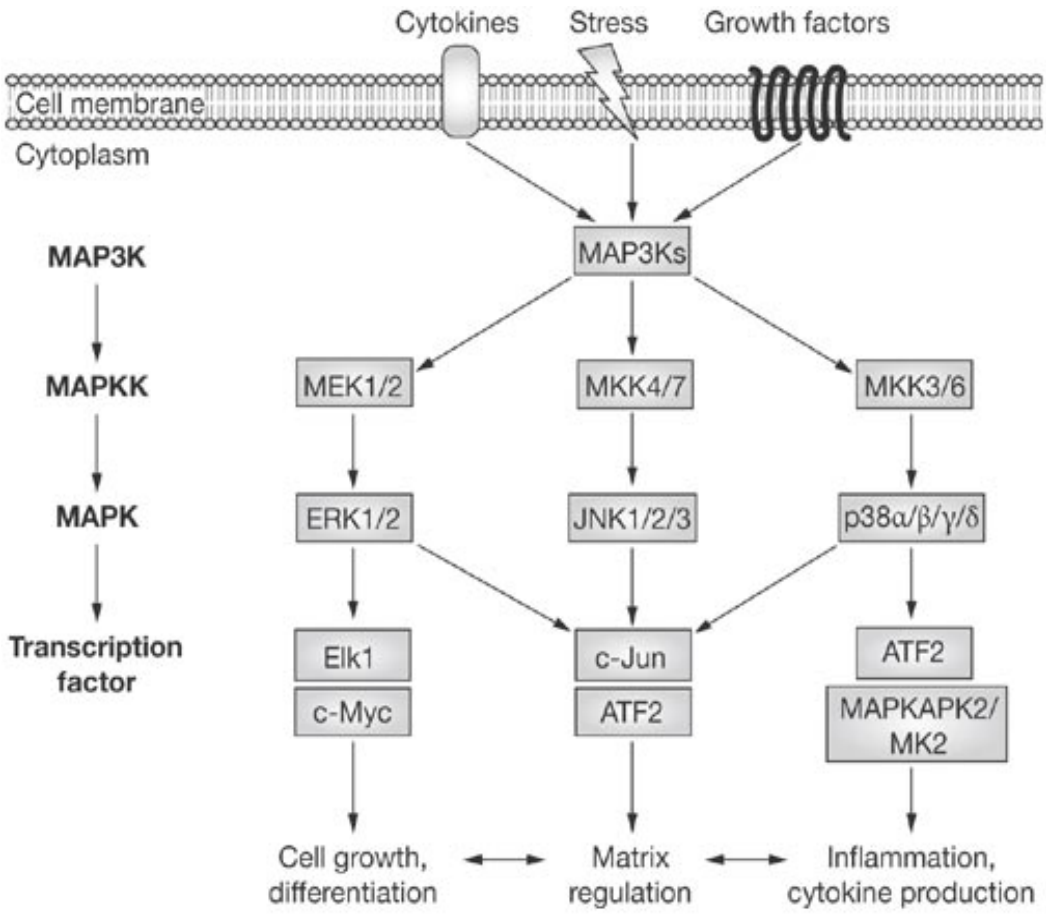

Figure 1.8. The MAPK signalling cascade.

The top tier shows the MAP3Ks, the second tier shows the MKKs, and the third tier comprises the MAPKs (ERK, JNK, p38) that regulate various genes through phosphorylation of transcription factors (e.g. c-Jun, ATF2) and other kinases (MAPKAPK2/MK2). The primary, but overlapping, responses include cell growth and differentiation (ERK), matrix regulation (JNK), and inflammatory cytokine production (p38). [Adapted from ref. (272)]

C. jejuni induces ERK and p38 activation in IECs leading to IL-8 production (249). In DCs, hyper-induction of ERK and p38 in the presence of *C. jejuni* flagella leads to enhanced IL-10 expression(218). Therefore, MAPK signalling is emerging as an important target in *C. jejuni*-induced pathogenesis.

1.3.2.7.3 Interferon-regulatory transcription factors (IRFs)

Interferon-regulatory transcription factors (IRFs) are a family of transcription factors involved in the regulation of type 1 interferon genes. IRF3 is downstream of TRIF-mediated signalling. *C. jejuni* induces IRF3 phosphorylation via TLR activation leading to the upregulation of IFN-β secretion (228). Although type 1 interferons are more commonly associated with viral infections, there is increasing evidence that IFN-β may enhance anti-bacterial activity through a positive feedback mechanism (280).

1.3.2.7.4 Phosphoinositide-3-Kinase (PI3K)

Phosphoinositide 3-kinase (PI3K)-dependent signalling is involved in a diverse array of cellular functions including growth, proliferation, survival, and motility. More recently, PI3K has been implicated in the dampening of pro-inflammatory NF-κB signalling in response to LPS (281). In a gnobiotic IL-10-/- murine model of infection, inhibition of mammalian target of rapamycin complex-1 (mTORC1), a downstream effector of PI3K activation, inhibits colitis in mice (282). Contrary to the LPS reports, mTORC1 inhibition decreases NF-κB activation although it does not ameliorate disease (71). Blocking mTORC1 inhibits production of IL-1β and IL-17, a potent neutrophil chemoattractant, leading to a decrease in neutrophil infiltration. In the same model, mTORC1 was shown to mediate *C. jejuni*-induced colitis by regulating NF-κB and pro inflammatory signalling (282). In addition, IL-10 production is regulated by the PI3K pathway which

antagonises the activation of glycogen synthase kinase 3 (GSK3), a negative regulator of IL-10 (283). Currently, the contribution of this pathway to the *C. jejuni*-induced IL-10 response remains unknown.

1.4 Hypothesis and Aims

Although our understanding of bacterial-gut immunity is improving, mechanisms involved in asympotomatic colonisation versus disease remains limited. To improve our understanding of these microbial-host interactions we hypothesised that the structural components of common enteric pathogens, such as *C. difficile* and *C. jejuni,* facilitate immune evasion by engaging inhibitory receptors on innate immune cells.

In this study we set out to investigate the immunogenic properties of the peptidoglycan moiety from *C. difficile* and the receptors and signalling mechanisms associated with those. We also wanted to study the role of bacterial moieties, such as the flagellum and LOS, in the immune evasion strategy employed by *C. jejuni.* Firstly, to better understand the way in which *C. jejuni* induces production of the anti-inflammatory IL-10 cytokine in human APCs via its flagellum. Secondly, to investigate potential interactions between Siglec receptors and *C. jejuni* and how they may affect the immune response to the pathogen.

Chapter 2

Materials and Methods

2.1 Peptidoglycan isolation and sonication

Peptidoglycan (PGN) from WT and an *lgt C. difficile* 630 isogenic mutant strain was extracted by Dr. Johan Peltier as detailed in Peltier et al. (2011) (156). Insoluble PGN was suspended in 3 ml sterile water at 5 mg/ml in a 5-ml glass tube and sonicated for 30mins (3x 10mins cycles with breaks for cooling in between cycles) in an ice-water bath with a 1/8 inch microprobe (70-80W output on a Vibra-Cell VCX400, Sonics sonicator). The solution was sterilised by heating at 90°C for 30mins and stored in solution at 4°C. Sterility was assessed by plating a drop of PGN on a nutrient agar plate and incubating at 37°C for 24h.

2.2 Bacterial strains and culture

2.2.1 *C. jejuni*

C. jejuni strains were routinely cultured on blood agar (BA) plates supplemented with Campylobacter selective supplement (Oxoid, Hampshire, UK) and 7% (v/v) horse blood (TCS Microbiology) under microaerobic conditions at 37°C for 48 hours. The hypermotile strain 11168H is a variant of NCTC11168 WT (193); 81-176 is a milk-borne outbreak isolate (221). 11168H flagellin (flaA) and capsule (kpsM) isogenic mutants and an 81-176 flaA isogenic mutant were obtained from the London School of Hygiene and Tropical Medicine Campylobacter Resource Facility (http://crf.lshtm.ac.uk/index.htm).

2.2.1.1 FITC-labelled *C. jejuni*

1 x 10^9 CFU/mL were mixed 1:1 with saturated Fluorescein isothiocyanate (FITC; Sigma) and incubated in the dark at room-temperature (RT) for 30mins with gentle

agitation. After incubation bacteria were washed three times before resuspension in phosphate buffered saline (PBS).

2.2.1.2 Heat-inactivated *C. jejuni*

C. jejuni cultures were harvested, washed and the optical density (OD) at 600nm was adjusted to 0.2 in PBS containing 2 mM $MgCl_2$. Samples were heat inactivated in a water bath at 56°C for 45mins.

2.2.1.3 *C. jejuni* flagella isolation and coupling to beads

Following growth at 37°C for 48 h, bacteria were harvested and resuspended in PBS. A volume of cells equivalent to an OD600 of 20 was resuspended in 1 ml PBS. Cells were vortexed vigorously at RT for 30mins to physically dissociate the flagella. The suspension was centrifuged at 20,000×g for 10mins at 4°C, the supernatant was collected and centrifuged a second time to remove all cellular debris. The resulting supernatant was spun at 4,000 g for 13mins in a spin column of 10,000 MWCO (molecular weight cut off). For coupling of flagella to beads, 0.5 ml of 2.5% suspension of Red Carboxyl-modified Latex fluorescent beads (2 μm; Sigma) were mixed with 200 μg of purified flagella in 0.4 mg/ml 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (ThermoScientific, MA, USA), 0.6 mg/ml N-hydroxysulfosuccinimide (NHS) (LifeTechnologies, California, USA) solution in 10% morpholino) ethanesulfonic acid (MES) buffer (LifeTechnologies) at RT for 2 h according to the manufacturer's protocol. Flagella-coupled beads were washed, resuspended in PBS and stored at 4˚C. To determine the purity of isolated flagella as well as coating efficiency, coated beads were eluted in sodium dodecyl sulfate (SDS) sample buffer and electrophoresed on a

polyacrylamide gel. Post electrophoresis, the gel was stained with Coomassie Blue for protein detection.

2.2.2 *Salmonella* Typhimurium

Bacteria were grown on agar plates (Sigma Aldrich) and kept at 4°C for a maximum of two weeks. For long-term storage single colonies were transferred to glycerol stocks and stored at –80°C. Prior to the infection, a single colony was grown in 10ml of Lysogeny Broth (LB) (Sigma Aldrich) in a 15ml Falcon tube at 37°C.

2.3 Cell line culture

2.3.1 Cell culture reagents

All plastic including 6, 12, 24, 96 well plates, 25cm^2, 75cm^2 tissue culture flasks, pipettes and polypropylene coated eppendorf tubes were purchased from Fisher Scientific, Leicestershire, UK. Cell culture reagents were purchased from Invitrogen, Paisley, UK.

2.3.2 Cell lines

Chinese hamster ovary (CHO) cells were cultured in complete Ham F12 media, supplemented with 10% heat inactivated FCS, 1% Penicillin/Streptomycin (Pen/Strep) and Non-Essential Amino Acids (NEAA). Cell monolayers were grown in 75cm^2 flasks at 37°C, 5% CO_2. Confluent cultures were washed with 1x PBS to remove serum, and incubated with trypsin/EDTA for a few minutes until complete detachment of the

monolayer. Cells were passaged at a 1:10 ratio in compete media. For long term storage cells were stored in freezing media (10% DMSO, 40% FCS, in DMEM).

Human embryonic kidney (HEK293) cells were cultured in DMEM +Glutamax, supplemented with 10% FCS, 1% Pen/Strep. Cell monolayers were grown in 75cm^2 or 175cm^2 culture flasks at 37°C, 5% CO_2. Confluent cultures were washed with 1x PBS to remove serum and detached as above. Cells were passaged at a 1:10 ratio in compete media.

Tohoku Hospital Patient-1 (THP-1) cells were cultured in RPMI-1640 + 1% L-Glutamine, supplemented with 10% FCS and 1% Pen/Strep. Cells were grown in 25cm^2 or 75cm^2 upright culture flasks at 37°C, 5% CO_2. Every 3 to 4 days - at approximately 80% confluency - cells where collected by centrifugation at 1,500 rpm for 5mins at RT and passaged at a 1:10 ratio using complete media. For dTHP-1 differentiation, cells were treated with 10ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h followed by 24h incubation in media alone prior to commencement of co-culture studies,

2.3.3 Co-culture studies

For bacteria – cell co-culture, *C. jejuni* cultures were harvested, washed and OD600 adjusted to 1 (=~3x10^9 cells). 1 x 10^6/mL PMA-differentiated THP-1 cells (referred as dTHP-1 cells hereafter) were cultured in RPMI containing 2 mM L-glutamine and 0.05% fetal calf serum (FCS), a day prior to infection with *C. jejuni* at an multiplicity of infection (MOI) 100. After inoculation, cells and bacteria were spun (1500 rpm, 5mins) to promote bacterial-cell association. Similar methodology was used for other bacteria.

2.4 Primary cell culture

2.4.1 Isolation of peripheral blood-derived monocytes

Blood from healthy donors (with appropriate consent) was collected in heparin-coated Falcon tubes and mixed 1:1 with RPMI-1640 media and then layered in falcon tubes containing Lymphoprep medium on a 3:1 ratio. Following centrifugation at 2,200 rpm for 25mins, peripheral blood mononuclear cells (PBMCs) were collected using a Pasteur pipette. The cells were washed twice in RPMI media, followed by a wash in MACS buffer (PBS + 2% FBS + 1 mM EDTA) and finally resuspended in 400µl of MACS buffer. For positive selection of monocytes, CD14 MACS MicroBeads were added, mixed thoroughly and incubated at 4°C for 45mins. Cells were then washed in excess MACS buffer and ran through a MACS LS Column attached to a MidiMACS Separator from Miltenyi Biotech (Bergisch Gladbach, Germany). The column was washed three times and then removed from the separator. CD14 positive monocytes were collected in a new collection tube by pipetting 5ml of MACS buffer into the column and applying a plunger. Alternatively, isolated PBMCs were cultured for 2h prior to selection of adherent monocytes. The cells were counted and platted in 6-well culture dishes in RPMI media supplemented with 10%FCS, 1% Pen/Strep.

2.4.2 *In vitro* differentiation of PBMCs into M1- and M2-macrophages

PBMC-derived monocytes were cultured in complete RPMI with 20ng/ml human GM-CSF or human M-CSF (Sigma-Aldrich, St. Louis, MO) to induce M1- or M2-type macrophage differentiation respectively for 5 days. Fresh differentiation media was added at day 3 and cells rested in complete RPMI for another 2 days.

2.5 Silencing of NOD expression by siRNA

PBMCs were transfected with scramble siRNA (sc-108066), human NOD1 siRNA (sc-37279) or human NOD2 siRNA (sc-43973) (Santa Cruz Biotechnology) using the Amaxa Nucleofector (Lonza). Briefly, PBMCs (1×10^6) were resuspended in 100µl of nucleofector solution followed by the addition of 8µg/mL of scramble, NOD1 or NOD2 siRNA, incubated at RT for 3mins, and nucleofected according to the manufacturer's instructions. PBMCs were then seeded on 12-well plates containing 1ml of RPMI supplemented with 10% autologous serum and incubated for 2 h at 37°C and 5% CO_2. M-CSF or GM-CSF were omitted so as not to influence the transcriptional profile further. After 2 h, adherent cells were washed and repleted with warm RPMI containing 20% autologous serum for 72 h. Transfected cells were used for subsequent experiments,

2.6 Lentiviral production

2.6.1 Plasmids

The eGFP pCMV plasmid was provided by Dr Holly Stephenson, IIIP program, Institute of Child Health. The NF-κB-luciferase SV40 plasmid was a kind gift from Dr David Escors, Infection and Immunity, University College London. Human WT NOD1-FLAG and human WT NOD2-FLAG pCMV vector plasmids were a kind gift from Dr Stephen E Girardin, University of Toronto, Canada. pRL Renilla luciferase reporter plasmid was purchased from Promega. Human Siglec-5 and human Siglec-9 pCMV6 cDNA clones were purchased from Origene, Rockville, USA.

2.6.2 Bacterial transformation

Competent *E. coli* DH5α bacteria were mixed with 500ng of plasmid DNA and incubated on ice for 30mins. Application of heat shock at 42°C for 45 sec, followed by a further 2mins on ice was done to promote DNA uptake. Transformed bacteria were allowed to recover by incubation at 37°C in 100µl of sterile LB broth for 1 h. Cultures were streaked on an agar plate with appropriate antibiotic (Table 2.1) and incubated overnight at 37°C to enable single colony growth.

Table 2.1 Plasmids and antibiotics used in transformation

Plasmid	Antibiotic	Antibiotic Conc. (/ml)
NF-κB	Ampicillin	100µg/ml
NOD1	Ampicillin	100µg/ml
NOD2	Kanamycin	50µg/ml

2.6.3 Isolation and purification of plasmid DNA

QIAprep spin midi-prep kits (Qiagen, California, USA) were used and plasmid DNA was isolated according to the manufacturer's instructions. DNA was eluted in 50µl distilled water.

2.6.4 Transformation of packaging cell line

The transfection mixture comprised of 7µg vector expressing construct (pHR_SIN), 3.5µg pMD.G2 (envelope genes helper vector) and 3.5µg pCMV-dR8.74 (gag/pol packaging genes helper vector) added to 1ml Opti-MEM media (Gibco) per flask. This was filter-sterilised (0.22µm filter) and added to 2ml Opti-MEM with 60µl Fugene 6 Transfection Reagent (Promega). The transfection mixture was incubated at RT for

15mins. 3ml were then added to a 175cm^2 flask seeded with 2x10^6 HEK293 cells in DMEM 24h in advance.

2.6.5 Virus harvesting and titration

HEK293 culture media was collected every 24h for three days, filtered and ultracentrifuged at 23,000 rpm for 2 h. The virus pellet was resuspended in 100µl PBS and aliquots stored at –80°C.

2.7 Cell transduction

2.7.1 Lentivector transduction of NF-κB reporter plasmid

THP-1 cells were transduced with lentiviruses (MOI 10) containing an NF-κB-luciferase reporter plasmid in the presence of 5 µg/mL protamine sulphate (Sigma Aldrich) and cultured for an additional 2 days. 1 x 10^5 transduced THP-1 cells (100µL RPMI, 10% FCS) were differentiated in the presence of 10ng/ml PMA for 24 h followed by 24h in media alone. dTHP-1 cells were stimulated with iE-DAP, MDP or PGN for the indicated times. Cells were equilibrated to RT, 100 µL Bright-Glo (Promega, Madison, WI, USA) reagent was added for 2mins before quantification of luciferase activity using a FluoSTAR Optima, (BMG Labtech, Germany) microplate reader.

2.7.2 NF-κB luciferase reporter assay

Dual NF-κB luciferase assay was carried out using Dual-Luciferase Reporter Assay System (Promega) according to the manufactures protocol. Briefly, cells were seeded in 24-well tissue culture plates one day before to ~70% confluence. Transfection mixes were prepared with 75ng NF-κB-luciferase plasmid, 30ng NOD1 or NOD2 plasmid and 50ng Renilla luciferase vector (the latter for normalization of transfection efficiency). All mixes were normalized to 300ng DNA per well in 60µl using an empty pcDNA3

expression vector. Polyethylenimine was added at 3µl per 1µg DNA ratio and the mix incubated at RT for 20mins before adding 0.7ml of DMEM. After 2 h, more media was added to a total volume of 3ml. 12 h after transfection, fresh media containing NOD ligands were added. After 8 h, media was aspirated, 100µl of lysis buffer was added and plates rotated gently for 15mins. The lysate was transferred onto white CELLSTAR microplates (Greiner Bio-One, Germany) and luciferase activity analysed using a FluoSTAR Optima, (BMG Labtech, Germany) microplate reader.

2.8 Bacterial infection assays

2.8.1 Gentamicin Protection Assay

2 x 10^5 dTHP-1 cells (RPMI/ 2 mM L-glutamine/ 10% FCS) were infected with WT and *ΔFlaA C. jejuni* 11168 MOI 100 for 4 h at 37ºC Post-infection, cells were washed twice with sterile PBS, followed by addition of RPMI containing 150 µg/mL gentamicin and incubation for a further 2 h to kill extracellular bacteria. Following washing, cells were lysed in 0.5 mL 0.1% Triton-X 100 (Sigma) in PBS for 5mins prior to serial dilution and plating. Colony forming units (CFU) were quantified.

2.8.2 Siglec-CHO adhesion assay

Human Siglec-5 or Siglec-9 clone plasmid (Origene) was ligated into an expression pDUAL lentivector plasmid; viral propagation was conducted in a HEK293T packaging cell-line as described above. CHO cells were transduced (MOI 10 or 100) and expression assessed by flow cytometry. 1x10^6 cells/mL were resuspended in F12 media containing 0.5% FCS. 300 µL of the re-suspension was co-cultured with FITC-labelled *C. jejuni* (MOI 100, 2 h, 4ºC to inhibit phagocytosis) in sterile polystyrene tubes with gentle rotation. For competition binding, cells were pretreated with 100ng *C. jejuni*

lipooligosacchride (LOS) for 1.5 h prior to co-culture with bacteria. After washing, cells were fixed in 4% paraformaldehyde (PFA). Bacterial adherence was assessed by flow cytometry using a FACSCalibur (BD Biosciences).

2.8.3 Siglec-10 overexpressing THP-1 cells

THP-1 cells were transduced as above. 2.5 x 10^5 /ml dTHP-1 cells (monocytic THP-1 cells differentiated in the presence of 50ng/ml PMA for 24h followed by 24h in media alone) were infected with *C. jejuni* strains at an MOI of 100. Cytokines were assessed 24h post-infection by ELISA (eBioscience).

2.9 Polymerase Chain Reaction

2.9.1 RNA extraction and cDNA synthesis

Cells pellets were lysed in Trizol Reagent (Invitrogen) (1ml per 1x10^6 cells). Chloroform (200µl) was added and samples were left to stand for 5mins at RT, then centrifuged at 12,000 rpm at 4^{o}C for 15mins. The aqueous phase of the separated mixture, containing the RNA, was collected and ice-cold isopropanol was added at a 1:1 ratio. The solution was left to stand for 10mins at RT, next RNA was pelleted by centrifugation at 12,000 rpm for 10mins at 4ºC. The pellet was washed twice in 75% ethanol, air-dried and re-suspended in 27µl nuclease-free water. Double-stranded DNA contamination in the samples was removed by using the Ambion TURBO DNA-free Kit (Invitrogen) according to the manufacturer's instructions. RNA concentration was measured by a Nanodrop reader (Thermo Scientific). RNA (2µg per reaction) was incubated at 65ºC for 5mins with oligo (dT1) and random (hexamer) primers (1µg per reaction) in PCR tubes and then chilled on ice for 2mins. For the reverse

transcription reaction, the following were added to each PCR tube: 4µl Bioscript Red buffer (5x), 10mM dNTPs, 1.5µl nuclease-free water, 0.5µl Bioscript enzyme in 10µl total volume and the reaction was carried out in a MyiQ Thermocycler (Biorad) with the following settings:

Step	Temperature (ºC)	Time
Primer extension	25	10mins
Reverse transcription	42	1hour
Inactivation	75	10mins

2.9.2 Semiquantitative-PCR

Primers were reconstituted in MilliQ water 100pmol final concentration and stored at –20ºC. Working solutions of 5 pmol/µl concentration were prepared and stored at 4ºC. For each reaction 5µl cDNA was mixed with 25µl Biomix Red 2x [1.5µM Mg^{2+}], 500nM primer mix and 15µl MilliQ water. Polymerase Chain Reaction (PCR) was carried out in a MyiQ Thermocycler (Biorad) as follows: initial denaturation 95ºC for 10 min (1 cycle); denaturation 95ºC for 30 sec, annealing 60ºC for 1 min, extension 72ºC for 45 sec (35 cycles), final extension 72ºC for 10mins. Samples were analysed by electrophoresis in a 2% agarose gel using Hyperladder IV (Bioline). Primers utilised: NOD1 fwd TCCAAAGCCAAACAGAAACTC, NOD1 rev CAGCATCCAGATGAACGTG, NOD2 fwd

CATGTGCTGCTACGTGTTCTC, NOD2 rev CCTGCCACAATTGAAGAGGTG, β-actin fwd CGCCCAGACTTCTGCATGG, β-actin rev GGATGACCTGACTCACAAACT.

2.9.3 Quantitative Real Time-PCR (QRT-PCR)

QRT-PCR reaction master mixes contained 10 µL SYBR Green (Invitrogen) and 5 pmol of forward and reverse primer. PCR was performed in duplicate in a Rotor-Gene 6000 machine (Qiagen) as follows: initial denaturation 95ºC for 10mins (1 cycle); denaturation 95ºC for 15 sec, annealing 58ºC for 30 sec, extension 72ºC for 30 sec (40 cycles). Rotor-Gene (Qiagen) software was used for data analysis. Primers utilized: IL-10 fwd GGTTGCCAAGCCTTATCGGA, IL-10 rev ACCTGCTCCACTGCCTTGCT, Polr2a fwd CGCCCAGACTTCTGCATGG, Polr2a rev GGATGACCTGACTCACAAACTG.

2.10 Protein analysis

2.10.1 Enzyme-linked immunosorbent assay

eBioscience Ready-Set-Go Elisa Kits were used according to the manufacturer's instructions and the procedure carried out in Maxisorp ELISA plates (NUNC). Samples were analysed in duplicate and the results obtained using a 96-well microplate reader (Bio-Rad). GraphPad Prism 5 and Microsoft Excel 2010 were used for graphical representation and statistics.

2.10.2 Recombinant Siglec Elisa

Live *C. jejuni* were harvested in PBS and OD600 adjusted to 0.2. 100µL of each bacterial strain was plated in triplicate in 96-well Maxisorp ELISA plates (NUNC Inc., the Netherlands). Plates were left open overnight at 37°C to allow bacterial adherence,

then washed three times in PBS/Tween (0.05%) and blocked in 1.5% milk powder for 2 h at RT. Recombinant Siglec-5-Fc or Siglec-9-Fc chimera (R&D Systems) were pre-complexed with peroxidase-conjugated anti-human IgG Ab (1:3000; Sigma) in 0.05% normal goat serum for 1 h with rotation. After washing, 100µL precomplexed Siglec-5-Fc or Siglec-9-Fc was added per well, the plates were incubated for 2 h at RT, washed four times PBS/Tween (0.05%) and developed using 100µL tetramethylbenzidine substrate (Biolegend) per well. After an appropriate incubation time (approx. 5 min), the reaction was stopped by adding 100µL of 2M H_2SO4 per well and signal intensity measured spectrophotometrically at 450nm in a 96-well microplate reader (Bio-Rad, the Netherlands).

2.10.3 Western Blotting

Cells were lysed in ice-cold lysis buffer (300µl per 1x106 cells) for 10mins. Protein concentration was assessed by a Bradford assay using Bradford Reagent (Sigma Aldrich). The manufacturer's protocol was modified for a 96-well plate assay and sample fluorescence measured using a 96-well microplate reader (Bio-Rad). After adjustment of protein concentration, an equal volume of 2x loading buffer was added and lysates were heated at 95°C for 3mins. Lysates were vortexed briefly and centrifuged to pellet debris. Cell lysates were separated on 10 – 12% gels.

Resolving gel buffer (4x) was prepared by dissolving 181.5 g of Trizma base in 850 mL of H_2O (pH 8.8) and Stacking gel buffer (4x) by dissolving 60.5 g of Trizma base in 850 mL of H_2O (pH 6.8) (for details, see Table 2.2). 30µl of each sample was loaded per well along with SeeBlue Plus2 Pre-Stained Standard ladder (Invitrogen). Gels were run at 120V for 1.5 h in the appropriate buffer (see Table 2.3). Blots were transferred onto Hyperbond-C membrane at 200mA for 70mins with the apparatus on ice to avoid overheating and efficient transfer. Peroxidase activity was detected using Amersham

ECL Western Blotting Advance Detection Kit (GE Healthcare). Membranes were blocked using 5% skimmed milk in 1x PBS for 1h at RT. Primary antibodies (Table 2.4) were used at 1:1000 dilution in 5% milk with 0.1% Tween 20 in PBS. HRP conjugated secondary antibodies were used at 1:1000 (anti-mouse) and 1:2000 (anti-rabbit) dilution.

Membrane was washed 3x with 0.1% Tween 20-PBS for 5mins each, the blot was incubated with secondary antibody (Polyclonal rabbit anti-goat Ig/HRP; Dako, Denmark; 1:2000) overnight at 4°C on a shaker. Blots were washed 3x with 0.1% Tween 20-PBS for 10mins. Toxins were detected using an enhanced chemiluminescence (ECL) reaction (Amersham ECL Western blotting detection reagent, GE Healthcare) followed by autoradiography film (Film CL-Xposure, Thermo Scientific, Loughborough, UK). The membranes were developed with an automated X-ray film developer (Autorad).

Table 2.2 Agarose gel for Western Blotting

Reagent	Resolving Gel (12%)	Stacking gel (4%)
H_2O	3.75 ml	6 ml
Acrylamide 30%	6 ml	1.5 ml
Resolving buffer	3.75 ml	
Stacking buffer		2.5 ml
Glycerol	1.5 ml	
*APS	50µl	50µl
**T.E.M.E.D	10µl	10µl

*APS = Ammonium Persulphate

**T.E.M.E.D = Tetramethylethylenediamine

Table 2.3 Buffers used in Western blotting

Buffer	Contents
Blocking buffer	5% skimmed milk in PBS + 0.1% Tween
Loading buffer	Laemmli buffer: 125mM Tris HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue.
Stripping buffer	Re-blot Plus Strong (Millipore)
Running buffer (10x)	Trizma Base (30.3g) + Glycine, 98% (144.1g) + SDS (10g) made up to 1L in H_2O
Transfer Buffer (10x)	Trizma Base (30.3g) + Glycine, 98% (144.1g) made up to 1L in H_2O

Table 2.4 Antibodies used in immunohistochemistry

Primary antibody	Clone	Manufacturer
CD14	M5E2	Biolegend
CD68	Y1/82a	Biolegend
ASC	aa2-27	LSBio
NOD1	ab22143	Abcam
NOD2	2D9	eBioscience
ERK1/2	137/F5	Cell Signalling
ERK1/2 (total)	9102	Cell Signalling
JNK	9552	Cell Signalling
p38	D13E1	Cell Signalling
EGF-R	pY1173 Alexa	eBioscience
SHP-2	pY542 Alexa	eBioscience
β-actin	AC-15	Invitrogen
Siglec-5	194128	R&D Systems
Siglec-9	191240	R&D Systems

2.10.4 Phopho-array analysis

Protein phosphorylation analysis was carried out using the Human Phospho-Immunoreceptor Array and the Human Phospho-Kinase Array kits (R&D Systems) according to the manufacturer's instructions. Pixel density analysis was performed using Gimp software.

2.10.5 Immunofluorescence

A 13-mm round glass coverslip was aseptically transferred into a well of a 24-well tissue culture plate. Approximately 1 x 10^4 cells were plated onto each coverslip. After incubation with media containing the appropriate stimuli, media was removed and the cells rinsed once in 1ml of PBS. Cells were fixed for 15mins in 4% PFA at RT prior to performing three 5mins washes with PBS with mild shaking. The coverslips were incubated with combined permeabilizing and blocking solution (3% BSA, 10% FBS and 0.2% Triton-x100 in PBS) for 1.5h at RT. Coverslips containing permeabilized and blocked cells were transferred to a light-protected chamber where solution of primary antibodies (see Table 2.4) was added to each (50 µl per coverslip) and incubated for 3-3.5h at RT or overnight at 4°C. Subsequently coverslips were transferred to a new 24-well tissue culture plate containing PBS to be washed as above. 50µl of secondary antibody solution was added to each coverslip and incubated for 1h at RT, followed by another washing step. Coverslips were allowed to air dry before being applied upside down facing a drop of Vectashield® mounting agent on a microscope glass slide. Round coverslips were sealed around the edge using a nail varnish solution. Slides were analysed using a Zeiss Axiovert 135 microscope or a Zeiss LSM 710 (Carl Zeiss, Germany) inverted confocal microscope. Microscopy images were analysed using ImageJ (NIH, USA).

2.10.6 Flow-cytometry

0.5-1x10^6 cells were aliquoted into each assay tube by volume. Each tube was washed twice in 2 ml FACS buffer. Cells were resuspended in 100 µl FACS buffer (PBS + 0.5% BSA) and non-specific binding blocked by incubation for 10mins at RT. For intracellular staining, cells were permeabilized by adding 1ml ice-cold 90% methanol slowly while gently vortexing. After washing, cells were fixed in 4% PFA for 10mins at RT and washed again twice. Samples were incubated with fluorochrome-conjugated primary antibody at the recommended dilution for 1 h at RT, followed by washing in 2ml FACS buffer. Cells were resuspended in PBS and analyzed on a FACSCalibur (BD Biosciences). Flow cytometry data were analysed in FlowJo version 7.

2.10.7 Caspase-1 assay

Caspase-1 activity was measured with a Green FLICA Caspase-1 Assay Kit (Immunochemistry Technologies, Minnesota, USA) according to the instructions provided by the manufacturer. Fluorescent signal was measured in a FACSCalibur (BD Biosciences).

2.11 Statistical analysis

Data were analysed using GraphPad Prism version 5 (San Diego, USA). Statistical significance was calculated by the paired Student's *t* test. Non parametric t-test (Mann-Whitney) was performed to compare groups in non-parametric data. Grouped analysis was performed by two-way ANOVA and Bonferroni post-test to compare replicate means by row and compare each column to all the other columns. Data considered significant only if probability was $p<0.05$.

Chapter 3

Pattern recognition receptor-mediated sensing of *C. difficile* peptidoglycan

3.1 Introduction

Peptidoglycan (PGN) is the major component of the Gram-positive as well as a minor component of the Gram-negative bacterial cell wall; crucial for maintaining the structural integrity of the cell membrane, as well as conferring resistance to antibiotics (148). Emerging evidence suggests that the role of PGN is multifaceted. It not only is an important structural entity but it is also a crucial participant in host-pathogen interactions that determine host health and disease status. This paradigm has emerged with the discovery of immune cell receptors that sense PGN-derived motifs(150). Conserved microbial structures, such as PGN, referred to as MAMPs are recognized by cellular PRRs that are present on professional APCs such as DCs and macrophages. The two major families of PRRs are the cell surface and endosomal TLRs and the cytoplasmic NLRs (18). Early studies identified TLR2, a member of the TLR family of PRRs, as the receptor for PGN (25). However, further investigations employing a lipoprotein negative isogenic mutant of *Staphylococcus aureus* revealed that it is the surface lipoproteins in the Gram-positive cell wall, and not the lipotechoic acid or PGN, that activate TLR2 (28),(29). Studies suggesting that lipoproteins from Gram-positive bacteria are indeed TLR2 ligands (284), and key activators of innate immunity have been described (285),(286).

The clostridia genus comprises ~40% of the human microbiome and as yet its interactions with the gastrointestinal (GI) mucosal immune system are not understood. A member of this genus, *Clostridium difficile* is an emerging enteropathogen and the leading cause of nosocomial and community-acquired infections in the elderly worldwide. To date, the majority of studies investigating *C. difficile*-host interactions have focused on the role of bacterial toxins on *C. difficile* associated gastroenteritis and diseases. At present very little is known of how *C. difficle* PAMPs interact with the innate immune system (138). Herein, PGN moieties from wild-type (WT) *C. difficile* 630

and its lipoprotein negative (Δlgt) mutant strain were employed to investigate their potential interactions with the human host. As TLR signalling is central to the innate immune response, we hypothesised that TLR activation by *C. difficile* lipoproteins may contribute to the immune outcome in response to this infection.

The only receptors for bacterial PGN identified to date are the cytoplasmic NOD receptors of the NLR family. Each receptor recognises a distinct PGN moiety; NOD2 recognises muramyl dipeptide (MDP), a PGN-derived muropeptide moiety common to the cell wall of all Gram-positive and Gram-negative bacteria (40), while NOD1 recognises diaminopoimelic acid (DAP), a component of Gram-negative and some Gram-positive bacteria (287),(46). NOD1 was recently shown to mediate *C. difficile* recognition, as well as the immune response to the pathogen in a mouse model (77), where NOD1$^{-/-}$ mice exhibited increased inflammation and lethality in response to *C. difficiile* infection. However, the study did not identify the specific bacterial ligands involved in this interaction. In 2011, the molecular structure of the vegetative cell wall was characterised by Peltier *et al.* (156). The authors found *C. difficile* PGN to possess a DAP residue at the third amino acid position of the peptide polymer cross-linking the sugar backbone. Although DAP is commonly found in Gram-negative PGN where Gram-positive bacteria contain a lysine residue, DAP-type PGN has been reported on other Gram-positive bacilli (288),(289). This unique characteristic may hold the key to *C. difficile* recognition by NOD1 and the consequent immune response. NOD activation by PGN motifs drives the induction of NF-κB, a key transcriptional regulator of inflammatory (pro- and anti) signalling, resulting in chemokine (e.g. IL-8) and cytokine (e.g. TNFα and IL-10) secretion (290).

In addition to the NOD family, MDP has been shown to activate two other NLR proteins: NALP1 and NALP3, although evidence remains limited. Upon activation,

these NLRs can lead to the formation of 'Inflammasome' complexes, large molecular assemblies that promote the maturation of IL-1β and IL-18 *via* caspase-1 activation and also trigger pyroptosis, a form of inflammatory cell-death (291). NALP3 inflammasome activation has been reported in response to MDP (99),(292) and lipoproteins from *S. aureus* (293). Another study reported that MDP-activated NOD2 interacts with and activates caspase-1, a component of the NALP-1 inflammasome (95). Activation of the Inflammasome by *C. difficile* toxins is now well-established (294),(122). At present the potential role of other *C. difficile* bacterial components, including PGN and lipoproteins in mediating inflammasome activation has not been investigated.

In this chapter we aimed to:

1. To investigate the effect of *C. difficile* cell wall lipoproteins on TLR2 activation.

2. To establish the potential role of the NOD receptors in *C. difficile* PGN recognition.

3. To study *C. difficile* PGN-mediated effects on macrophage signalling pathways.

3.2 Results

3.2.1 *C. difficile* PGN-associated lipoproteins activate TLR2

For every part of this study both cell lines and primary cells were employed sequentially. Initially, the epithelial cell line HEK293, which is often utilised in PRR signalling studies as it lacks native expression of these receptors, followed by a cell line model of human macrophages as well as primary macrophages from healthy donors. The PGN moieties from WT *C. difficile* 630 strain and its *lgt* isogenic mutant were isolated and kindly provided by Dr J. Peltier (Imperial College, London). Post-extraction, freeze-drying lyophilisation led to PGN aggregation which required ultrasonication for re-suspension. Ultrasonication aimed to promote breakdown of insoluble PGN into releasing soluble fragments of high molecular weight. Soluble fragments must contain ligands for PRRs such as TLRs and NLRs. Firstly, it was important to establish the effect of sonication on PRR stimulation. Transfected HEK293 cells stably expressing TLR2, the receptor for bacterial PGN polymers were employed in this series of experiments. PGN was sonicated according to the most widely adopted methodology (295). PGN from the Gram-positive bacterium *S. aureus* was studied in parallel. *S. aureus* PGN has been studied extensively and is commercially available as an insoluble preparation. It is also a known trigger for TLR2-mediated HEK293 cell activation and therefore was considered an appropriate control (296),(28). TLR2 activation was assessed by measuring IL-8 chemokine, one of the main epithelial chemokine mediators, in the supernatants. *C. difficile* and *S. aureus* PGN preparations were sonicated for 15 and 30mins respectively prior to co-culture with untransfected and TLR2-transfected HEK293 cells. 24h post-stimulation, IL-8 protein was measured in supernatants by ELISA (Figure 3.1). Unsonicated PGN mediated an IL-8 response of ~ 250 and 450pg/ml respectively. Sonication had a marked effect on TLR2

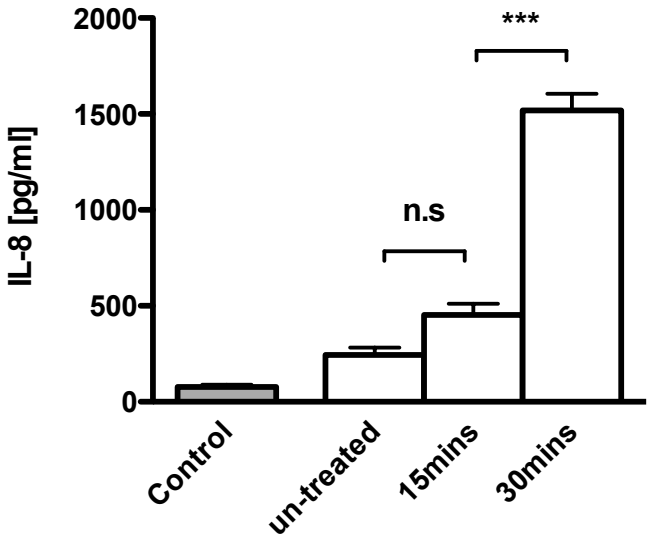

Figure 3.1. Sonication increases peptigoglycan-mediated TLR2 activation.

C. difficile peptidoglycan (PGN) polymer *was* sonicated for 15 and 30mins. TLR2-transfected HEK293 cells were stimulated with untreated and treated PGN (1mg/ml). 24h post-stimulatiion, IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (***P<0.001).

engagement as a statistically significant increase in IL-8 was observed in response to *C. difficile* PGN 30mins post-sonication ($P<0.01$). These experiments highlighted the requirement for PGN sonication prior to co-cultures. Based on this data, PGN preparations were routinely sonicated for 30mins prior to usage.

Next, we wanted to establish dose and time-dependent effects of PGN on host responses. Untransfected and TLR2-transfected HEK293 cells were stimulated with sonicated PGN at varying dosage at 6 and 24h. A dose-dependent increase in IL-8 protein was recorded in response to *C. difficile* PGN. At 6h, results were comparable between PGN from both species. Secreted IL-8 could be detected as early as 6h in response to 0.1μg/ml of PGN, with a dose-dependent increase in cells treated with 1μg/ml (Figure 3.2A). An increase in concentration from 1 to 10μg/ml showed a modest increase, most likely indicating near saturation of TLR2 signalling. The 1μg/ml dose was therefore considered to be most appropriate for further studies in HEK293 cells. IL-8 levels increased further 24h post-stimulation (Figure 3.2B). It was interesting to note that *C. difficile* PGN was a significantly more potent inducer of IL-8 in comparison to *S. aureus* PGN ($P<0.02$).

The capacity of *C. difficile* PGN to activate TLR2, as others have demonstrated for PGN from other bacteria was examined next. At the same time, we wanted to test the purity of our preparation for the presence of other TLR-activating contaminants. A range of TLR-transfected HEK293 cells were treated with our PGN preparations. As expected, only TLR2 and TLR2/6 (HEK293 cells became activated (Figure 3.3B and C), showing statistically significant increase in in IL-8 secretion both after 6h (TLR2; $P=0.0004$, TLR2/6; $P=0.0011$) and 24h (TLR2; $P=0.0013$, TLR2/6; $P=0.0027$). In contrast, WT, TLR4- and TLR5-transfected HEK293 cells were unresponsive to WT *C. difficile* PGN (Figure 3.3A, D and E) indicating the absence of ligands for these

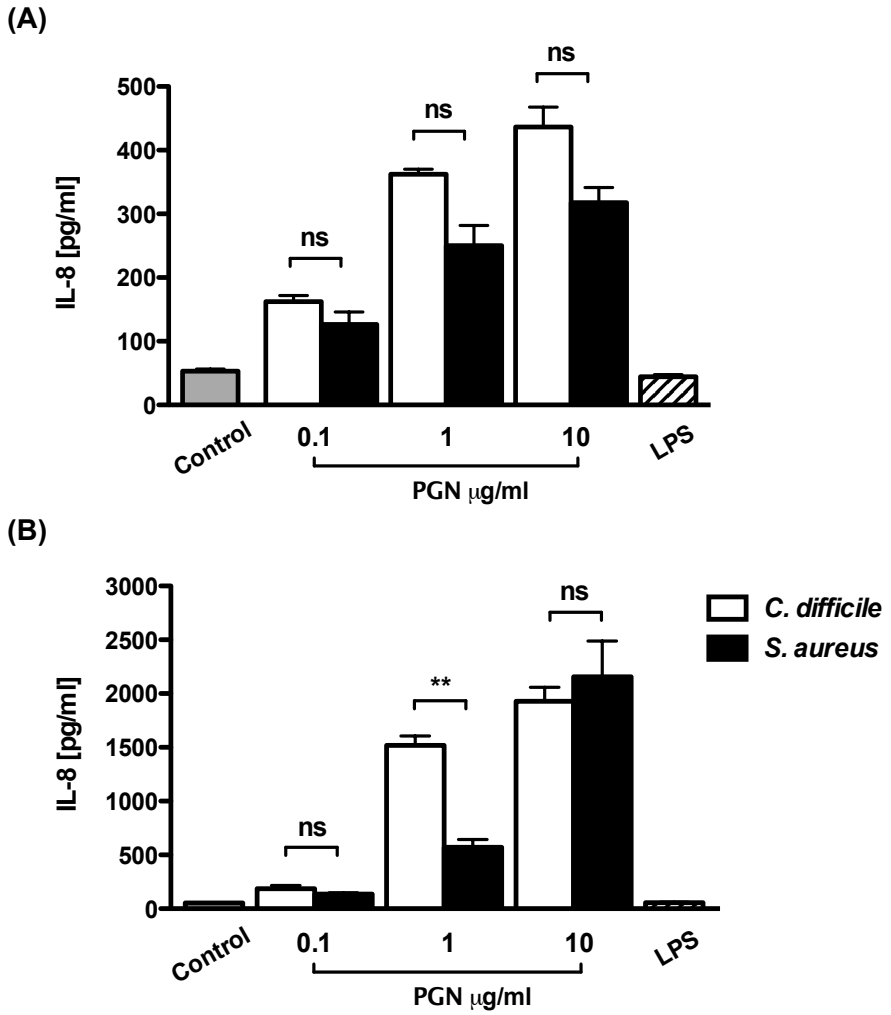

Figure 3.2. Dose- and time-dependent effects of *C. difficile* PGN on TLR2 activation.

TLR2-, CD14-transfected HEK293 cells were stimulated with PGN from *C. difficile* or *S. aureus* at increasing concentrations (0.1, 1 and 10μg/ml) for (A) 6h and (B) 24h. Stimulation with 10ng/ml lipopolysaccharide (LPS) from *E. coli* served as a negative control. IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (**P<0.01).

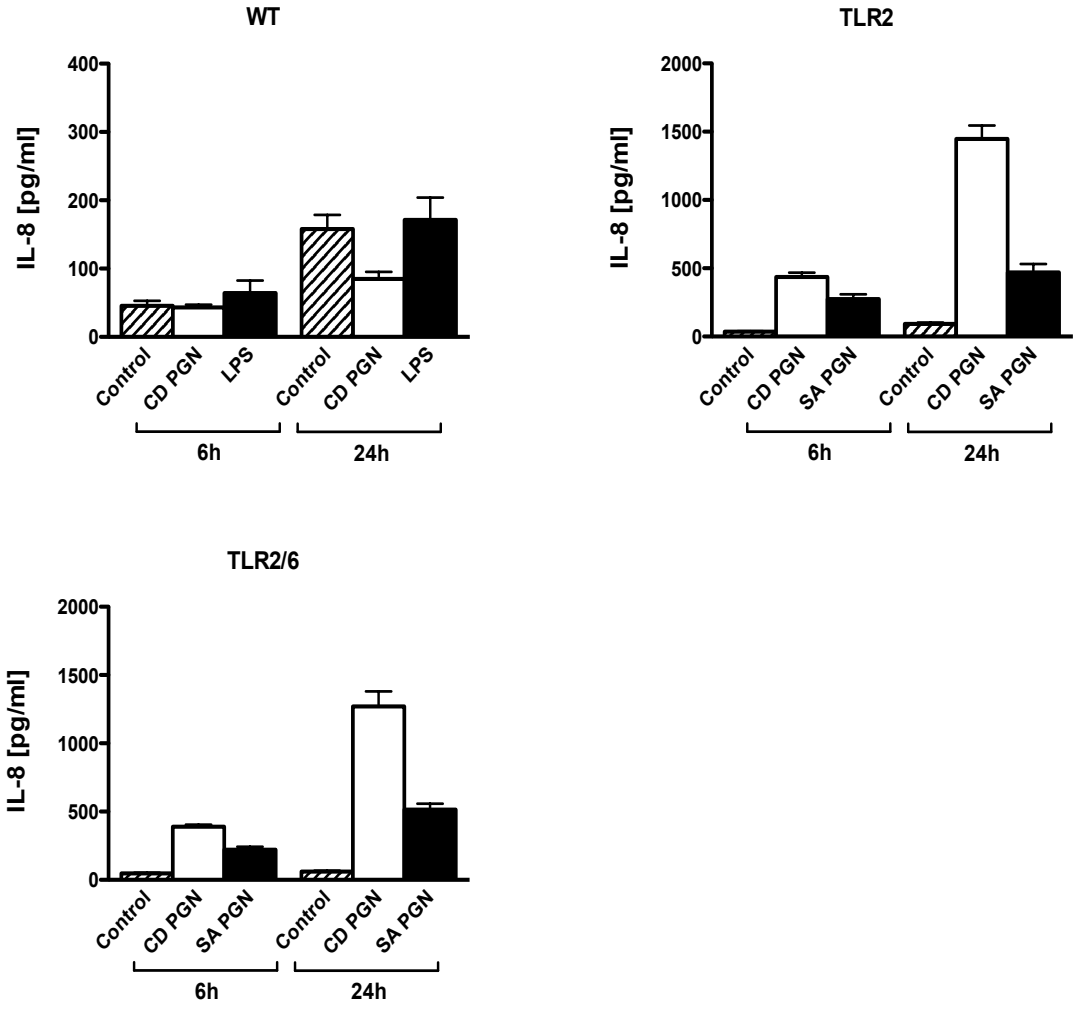

Figure 3.3. *C. difficile* PGN activates TLR2 and TLR2/6.

(A) WT and (B-E) TLR-specific HEK293 cells were treated with 1µg/ml *C. difficile* WT PGN for 6 and 24h. 10ng/ml *E. coli* LPS, 1µg/ml *S.aureus* PGN or 1µg/ml *Salmonella* typhimurim flagellin stimulation served as positive controls as indicated. IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from three independent experiments analysed in duplicate.

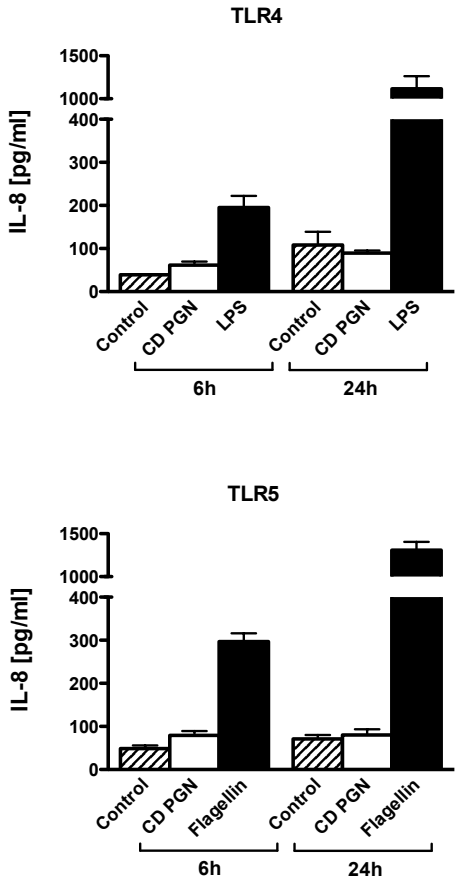

Figure 3.3. *C. difficile* PGN activates TLR2 and TLR2/6 (cont.).

(A) WT and (B-E) TLR-specific HEK293 cells were treated with 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN for 6 and 24h. 10ng/ml *E. coli* LPS, 1µg/ml *S.aureus* PGN or 1µg/ml *Salmonella* typhimurim flagellin stimulation served as positive controls as indicated. IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from three independent experiments analysed in duplicate.

receptors. The TLR2/6 HEK293 cells express a heterodimeric receptor composed of TLR2 and TLR6 monomers that is nevertheless able to recognise TLR2 ligands and as a result activate the same downstream signalling effectors(31).

Next, we looked at the effect of *C. difficile* PGN treatment on the key signalling mediator downstream of TLRs; the NF-κB transcription factor. For studies in PGN-mediated signalling, the widely used monocytic THP-1 cell line was used. The cell line was established from a patient with acute monocytic leukaemia in Japan(297). Previous studies in THP-1 cells have shown expression of functional TLRs and responsiveness to PGN(290). However, these cells occupy an early time point along the monocytic cell lineage; meaning a host of receptors involved in immune surveillance are expressed either at low levels or not at all. To create a better model of mature APCs, the cells are treated with PMA in order to induce differentiation into a macrophage-like phenotype (differentiated THP-1; dTHP-1) (234). To confirm the adoption of a macrophage-like phenotype following PMA treatment, cells were immunostained for the monocytic marker CD14 and the macrophage marker CD86(298). Analysis of dTHP-1 cells by flow-cytometry showed down-regulation of CD14, while CD86 was up-regulated (Figure 3.4), which is consistent with the maturation of primary monocytes towards the macrophage lineage(299) and, therefore, provides an appropriate model for our studies. A reporter cell line for NF-κB transcription activity was constructed using the THP-1 cell line. THP-1 cells were stably transduced with a plasmid carrying the NF-κB response element that drives the transcription of the luciferase reporter gene and intracellular luciferase levels quantified using a substrate of the enzyme. Again, PGN from *S. aureus,* known to activate NF-κB amongst other transcription factors(290), was used as positive control. Treatment of dTHP-1 cells with PGN led to a dose-dependent increase in NF-κB-mediated transcription (Figure 3.5). This was similar in cells treated with both PGN preparations

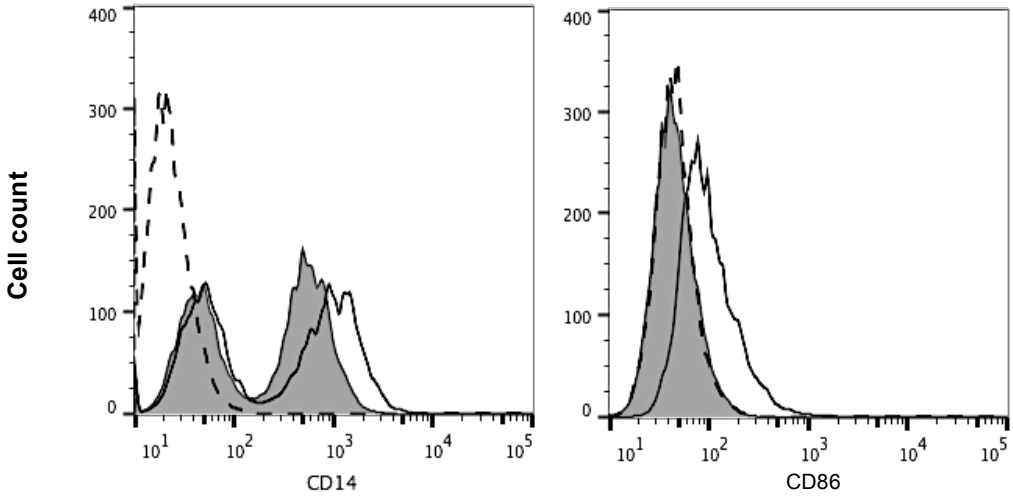

Figure 3.4. Phorbol 12-myristate 13-acetate (PMA)-treatment induces differentiation of monocytic -1 cells to macrophage-like phenotype.

Monocytic THP-1 cells were treated with 10ng/ml phorbol 12-myristate 13-acetate (PMA) for 24h followed by culture in complete RPMI for 24h. 48h after treatment, THP-1 (black line) and PMA-treated dTHP-1 (solid grey line) cells were stained with conjugated antibodies against the CD14 monocytic and the CD68 macrophage marker or isotype control (dotted line) and analysed by flow cytometry. Histograms are representative of three independent experiments performed in duplicate.

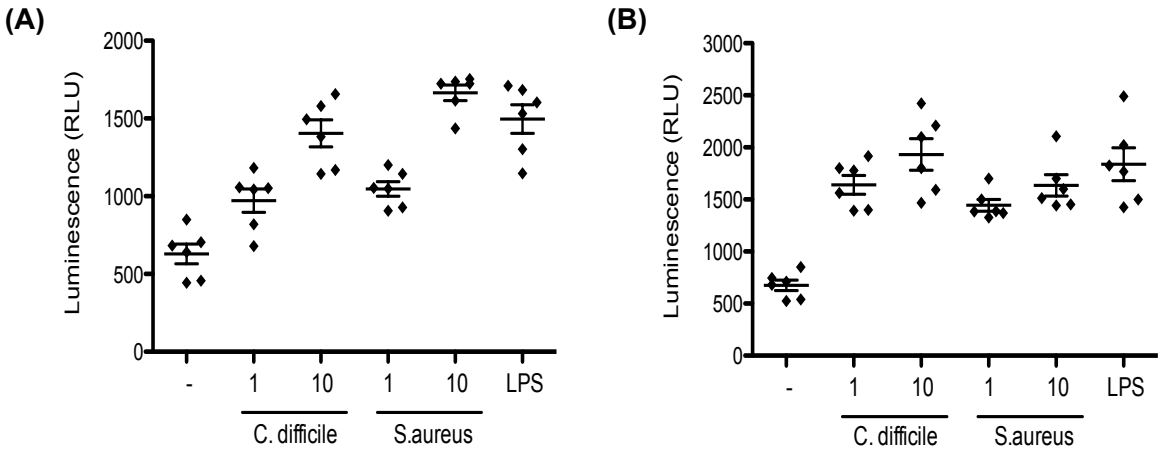

Figure 3.5. *C. difficile* PGN induces NF-κB activation.

Differentiated THP-1 cells (dTHP-1 cells) were transduced with NF-κB-firefly luciferase reporter and stimulated with 1 and 10μg/ml *C. difficile* or *S. aureus* PGN for (A) 6 and (B) 24h. Stimulation with 10ng/ml LPS from *E. coli* served as a positive control. Luciferase activity was determined by spectrophotometry (450 nm) (Values represent mean ± SEM from three independent experiments performed in duplicate).

for 6h (Figure 3.5A), but higher in those treated with *C. difficile* PGN after 24h (Figure 3.5B). As discussed earlier, TLR2 and NF-κB activation is mediated by lipoproteins found in cell wall heteropolymers rather than PGN (30). To establish whether lipoproteins in our preparation were the inducers of TLR2-mediated IL-8 secretion, a PGN preparation from the Δlgt *C. difficile* mutant lacking the lipoproteins was used. Treatment with WT but not *lgt C. difficile* PGN led to a TLR2 and TLR2/6-mediated IL-8 response (Figure 3.6), conclusively showing that lipoproteins are the TLR2 ligands present in *C. difficile* PGN. NF-κB induction was investigated using the NF-κB reporter cell line described earlier and was found to be significantly lower in *lgt* compared to WT *C. difficile* PGN-treated cells 4h post-stimulation (P=0.0195) (Figure 3.7). Between 6 and 24h post-stimulation no difference was noted between WT and *lgt* PGN indicating the effect of *lgt*–mediated signalling is an early event.

3.2.2 NOD-mediated recognition of *C. difficile* PGN

The evidence of TLR2-independent NF-κB activation led us to hypothesise that *C. difficile* PGN is recognized by other PRRs with the NOD proteins being the strongest candidates. To investigate the sensing of *C. difficile* PGN by these receptors, HEK293 cells were transiently transfected with plasmids expressing NOD1 or NOD2 together with an NF-κB-firefly luciferase and renilla luciferase reporter plasmid. HEK293 cells were cultured in transfection media for 4h, before being stimulated with 10μg/ml PGN for 16h. As a positive control, NOD1- and NOD2-transfected cells were treated with 10μg/ml iE-DAP or MDP respectively. At the end of the experiment, the cells were gently lysed by passive shaking and cell lysates transferred to a 96-well plate. Using an optic luminometer with injection capability, the substrates for firefly and renilla luciferase were introduced in the samples in succession. The light released from each

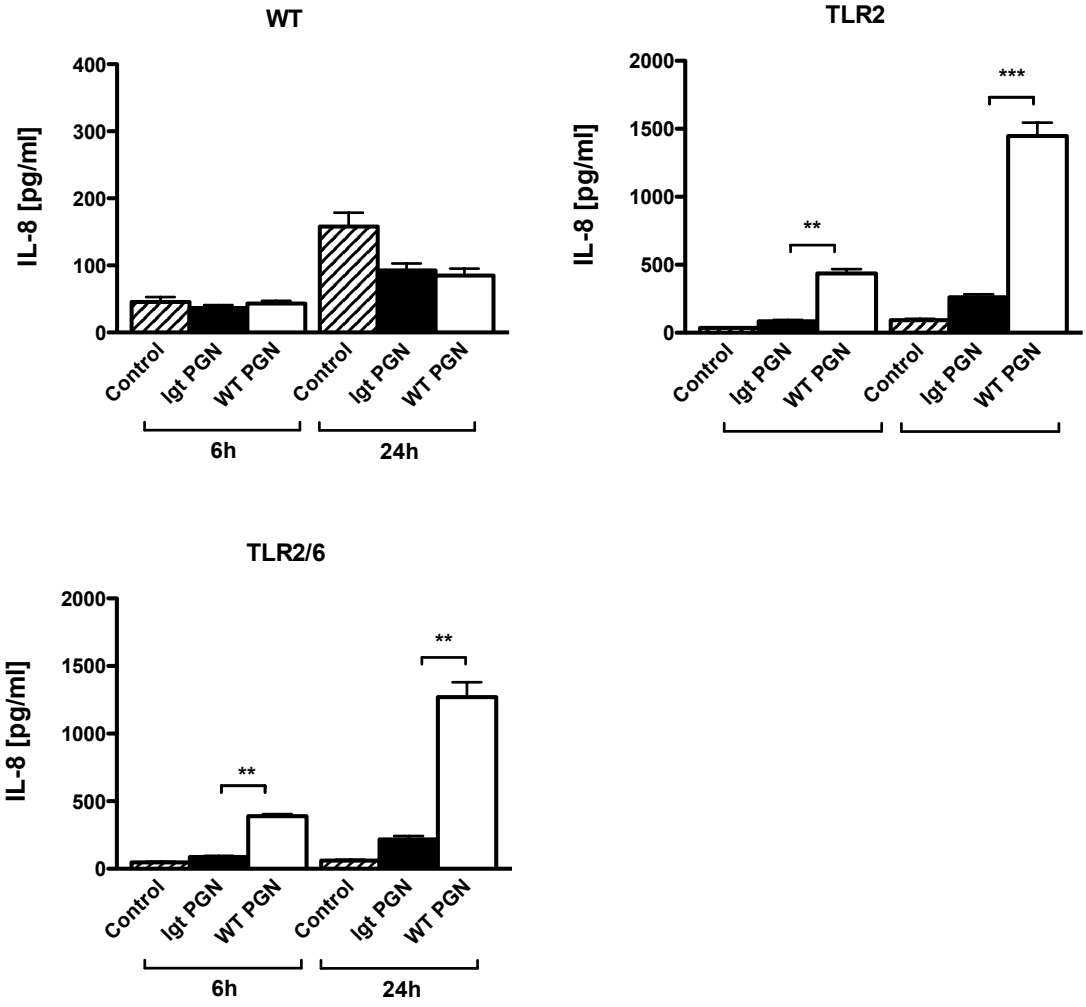

Figure 3.6. *C.* difficile cell wall lipoproteins activate TLR2.

(A) WT, (B) TLR2- and (C) TLR2/6-specific HEK293 cells were treated with 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN for 6 and 24h. IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from three independent experiments analysed in duplicate. Statistical analysis by paired t-test (**P<0.005, ***P<0.001).

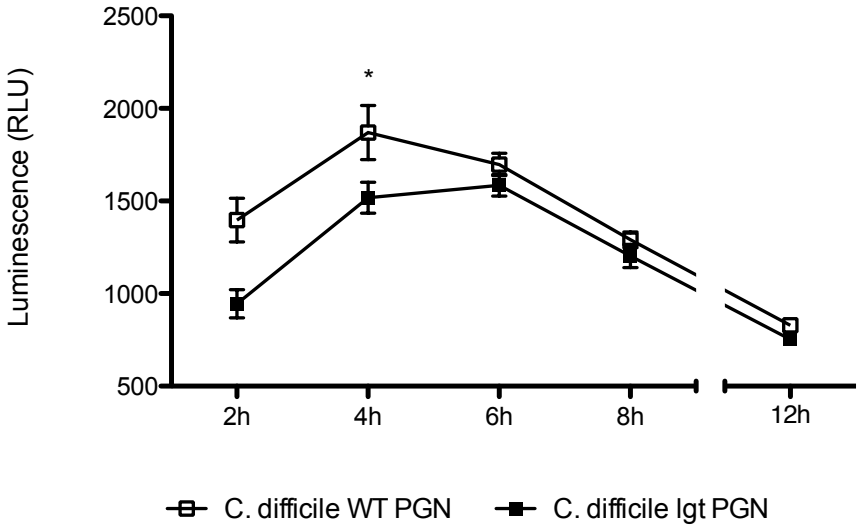

Figure 3.7. *C. difficile* lipoprotein- and PGN-mediated NF-κB activation.
dTHP-1 cells were transduced with NF-κB-firefly luciferase reporter and stimulated with 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN. Cells were lysed and luciferase activity was determined at the indicated time intervals by spectrophotometry (450 nm). Values represent mean ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

catalytic reaction was measured and experimental results normalised using the renilla luciferase O.D readings, according to the manufacturer's protocol. In HEK293 cells transduced with an empty vector plasmid only baseline luciferase levels were detected after PGN treatment (Figure 3.8). Interestingly, both NOD1 and NOD2 were able to mediate NF-κB activation in the presence of *C. difficile* PGN, with induction levels reaching statistical significance for both receptors (NOD1; $P<0.0001$, NOD2; $P=0.0004$). NF-κB activation was markedly higher in cells treated with the NOD ligands iE-DAP and MDP, affirming the experimental results.

Again, macrophage-like dTHP-1 cells were employed to further study PGN sensing as they have been shown to express NOD proteins in relatively high levels (300). To confirm this in our cell line, the impact of PMA treatment on the NOD1/NOD2 transcript and protein levels was analysed by quantitative real-time PCR and Western blotting respectively. Gene transcription (Figure 3.9A) and protein expression (Figure 3.9B) of both receptors was up-regulated in dTHP-1 cells, in agreement with previous reports (301).

To further investigate the role of NOD1 or NOD2 in the immune response to PGN, siRNA technology was used to silence the expression of the two receptors. Primary monocyte-derived macrophages (MDMs) were used in this study as they are known to express both receptors (299) and are able to mount a cytokine response to PGN (302). Freshly isolated PBMCs were transfected by electroporation with constructs for scrambled, NOD1- or NOD2-specific siRNA and plated in tissue culture dishes. A separate cell population was transfected with GFP to monitor transfection efficiency. Monocytes were selected by adhesion and differentiated into MDMs in 20% autologous serum for 72h. Over 60% of monocytes were GFP-positive after 24h as assessed by flow cytometry (Figure 3.10A). Cell enumeration by trypan blue staining

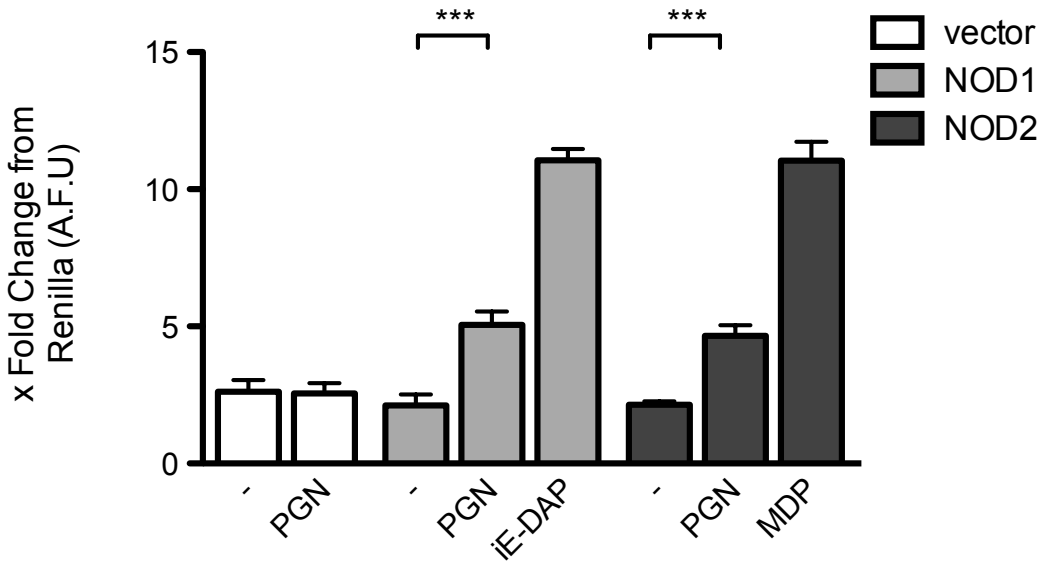

Figure 3.8. *C. difficile* PGN triggers NOD1- and NOD2-mediated NF-κB activation. HEK293 cells were transfected for 24h with vector, hNOD1 or hNOD2 plasmid, together with NF-κB-firefly luciferase and renilla luciferase reporter plasmid. After 24h, cells were stimulated with 10µg/ml *C. diffiicile* PGN. 10µg/ml g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) or muramyl dipeptide (MDP) stimulation served as a positive control. Luciferase activity was measured 16h after stimulation and firefly luciferase normalized to renilla luciferase levels. Data shown as fold change of firefly from renilla luciferase activity expressed as means ± SEM of three independent experiments performed in triplicate. Statistical analysis by paired t-test (***P <0.001).

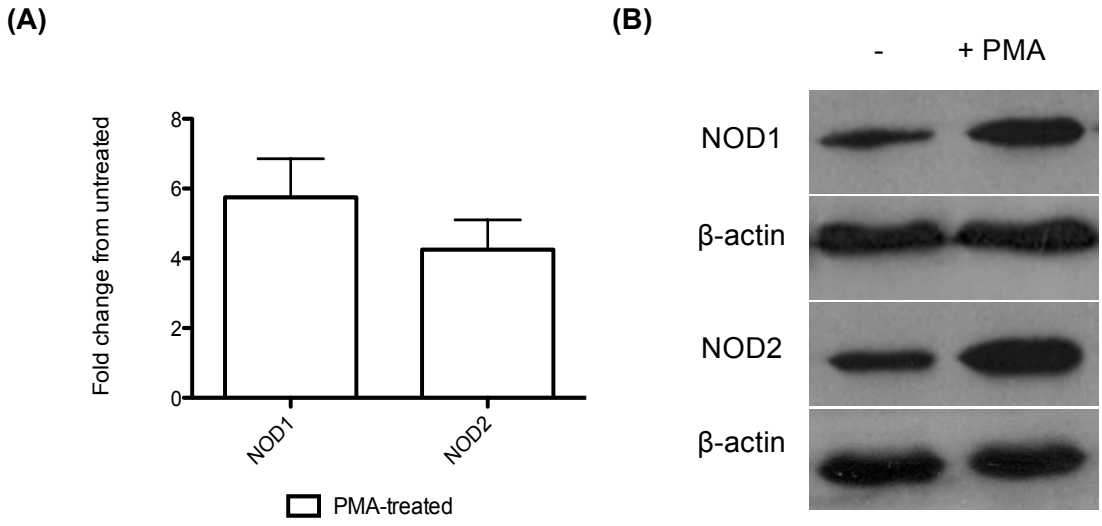

Figure 3.9. PMA-treatment leads to up-regulation of NOD1 and NOD2 in dTHP-1 cells.

THP-1 cells were treated with 10ng/ml PMA for 24h followed by culture in complete RPMI for 24h. 48h after treatment, NOD1 and NOD2 (A) gene transcription was analysed by quantitative RT-PCR (values represent mean ± SEM from three independent experiments) and (B) protein expression analysed by Western blot (representative blot from two independent experiments is shown).

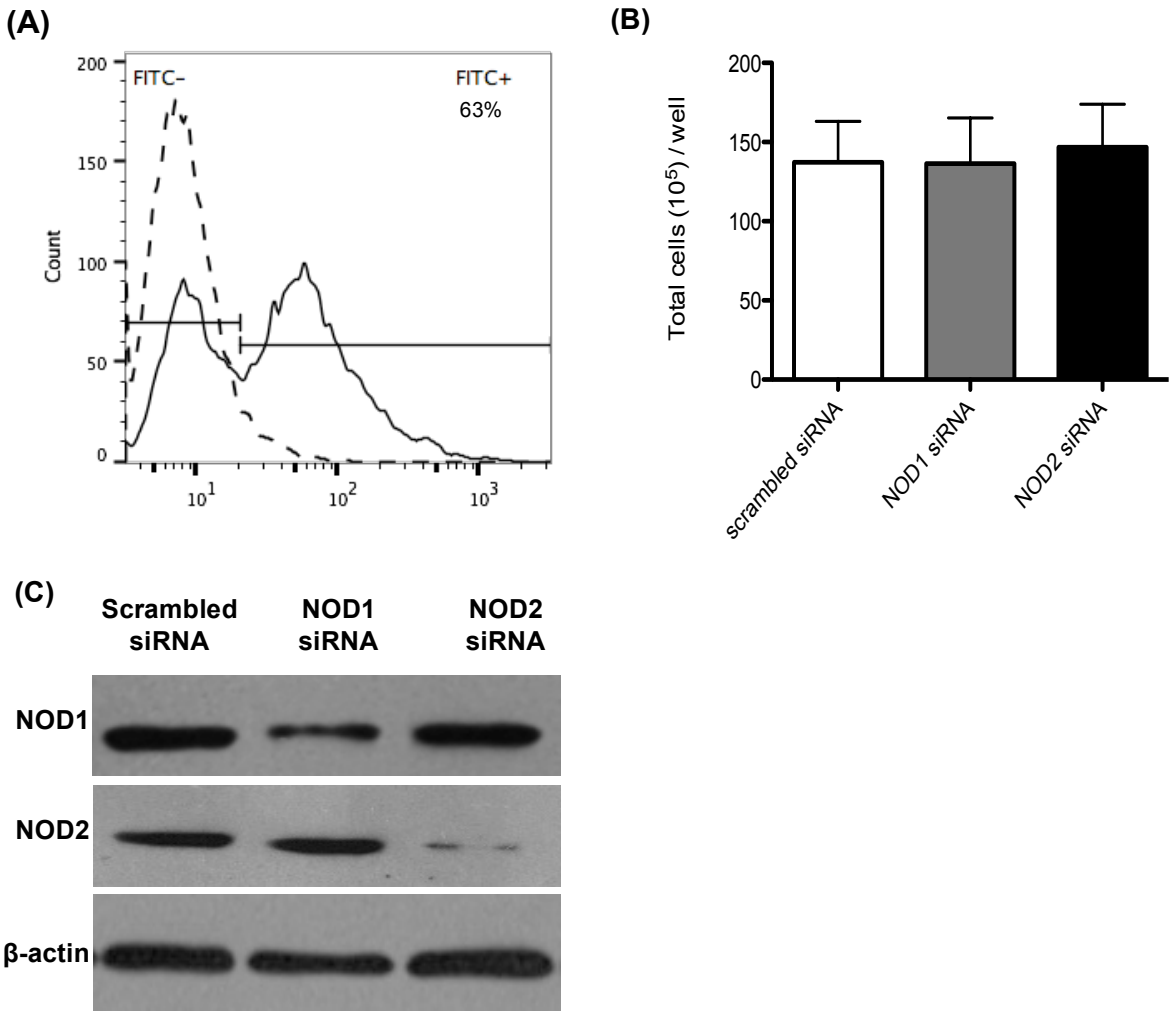

Figure 3.10. Knockdown of NOD1 and NOD2 expression in human monocyte-derived macrophages (MDMs) by siRNA methodology.

$1x10^6$ monocyte-derived macrophages (MDMs) were transfected with either a GFP, scrambled siRNA, NOD1 or NOD2 siRNA plasmid using an Amaxa nucleofector and plated in RPMI with 10% autologous serum for 72h. (A) Transfection efficiency was assessed by enumeration of GFP-positive cells by flow cytometry from three separate donors. (B) Cell death was assessed 72h post transfection to ensure equal numbers of cells by trypan blue staining (cumulative data shown as mean ± SEM). (C) After 72h, MDMs were lysed and NOD1 and NOD2 protein levels analysed by Western blot (representative blot from three different donors). Data shown are representative of three independent experiments.

72h post nucleofection demonstrated equal cell numbers amongst transfected MDMs, (Figure 3.10B). At 72h, NOD1/NOD2 knockdown was confirmed at the protein level by Western blotting (Figure 3.10C). Next, NOD1/NOD2 siRNA transfected cells were stimulated with 10μg/ml MDP or iE-DAP respectively to verify loss of function. Stimulation of NOD1 siRNA-transfected MDMs with iE-DAP led to a significant decrease in the TNFα response compared to cells transfected with scrambled siRNA (Figure 3.11). Likewise, MDMs transfected with NOD2 siRNA were almost completely unresponsive to MDP stimulation. Collectively, these data demonstrate the loss of NOD1 or NOD2 expression and functionality in transfected MDMs. Finally, cells were stimulated with 10μg/ml PGN for 24h and secreted TNFα measured by ELISA. No difference in TNFα secretion was observed between PGN-treated untransfected and scramble siRNA-transfected MDMs (Figure 3.12). Stimulation of either NOD1 or NOD2 knock down cells had a significant impact on cytokine expression (NOD1; $P<0.03$) (NOD2; $P=0.074$). Our data suggest that both NOD receptors are involved in mediating the cytokine response to *C. difficile* PGN.

3.2.3 *C. difficile* PGN-induced signalling in human macrophages

Post NF-κB-mediated signalling, activation of the inflammasome is a second component of a two-step process that leads to the secretion of a key pro-inflammatory cytokine IL-1β (291). Various danger and stress signals, including PGN-derived MDP motifs have been shown to trigger inflammasome activation in murine model systems (99). PGN-NOD2 mediated inflammasome activation in human innate immune cells has not been characterised. We postulated that *C. difficile* PGN may act as an inflammasome activator. Caspase-1 activation was assessed by intracellular staining for active caspase-1 using a fluorescent tag inhibitor. dTHP-1 cells treated with 1μg/ml

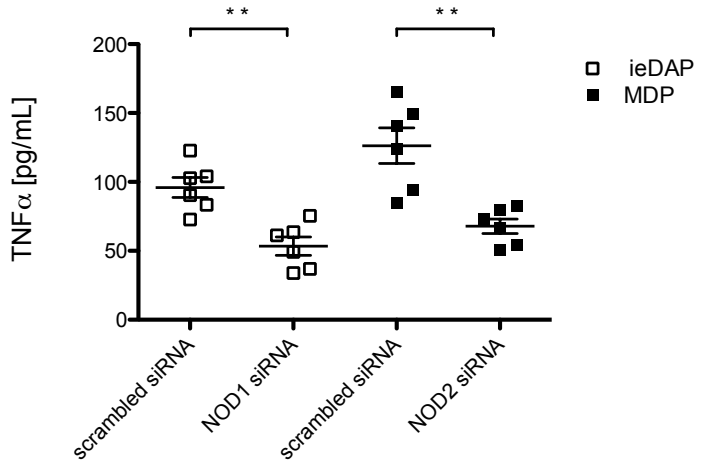

Figure 3.11. MDM TNFα protein expression in the presence of NOD1 and NOD2 siRNA.

2.5 x10^5 scrambled or NOD1/NOD2 siRNA-trasfected MDMs were stimulated with 10µg/ml iE-DAP or MDP. 24h post-stimulation, TNFα in the supernatant was quantified by ELISA. Values represent mean ± SEM from six individual donors. Statistical analysis by paired t-test (**P<0.01).

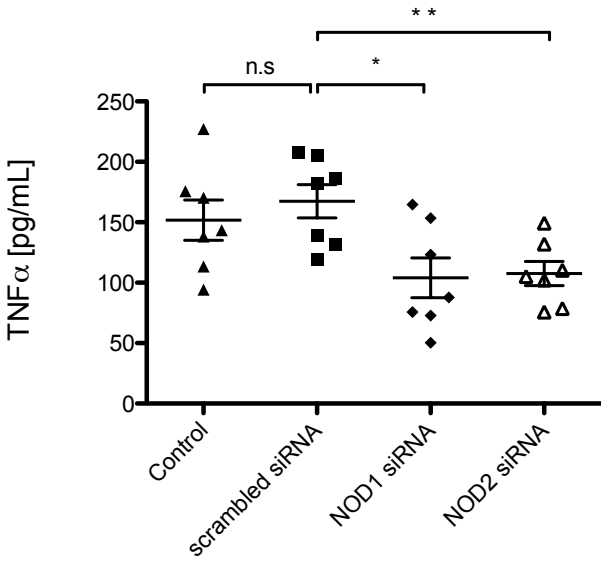

Figure 3.12. NOD1 and NOD2 mediate the *C. difficile* PGN-induced TNFα response in human MDMs.

Untransfected and scrambled, NOD1 and NOD2 siRNA-transfected MDMs were stimulated with 10µg/ml *C. diffiicile lgt* PGN for 24h and TNFα cytokine was measured in the supernatant by ELISA. Values represent mean ± SEM from seven individual donors measured in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, n.s P=0.069).

PGN or MDP for 4h were not fluorescently labelled as assessed by flow cytometry, indicating no active caspase-1 was present (Figure 3.13A). Activated caspase-1 was only observed in cells treated with LPS and ATP, which is a very well characterised inflammasome activator (303). NALP-1 and NALP-3 inflammasome activation was assessed by quantifying polymerisation of the ubiquitous inflammasome component ASC and caspase-1. Following treatment with 1µg/ml WT or *lgt* PGN, 1µg/ml MDP or LPS and ATP served as a positive control. 4h post-stimulation, dTHP-1 cells were immunostained with an antibody against ASC. In activated cells, diffused ASC should rapidly relocate into a punctate speck that can be easily visualised under a laser microscope (Figure 3.13B). By quantifying specks we concluded that treatment with *C. difficile* PGN did not lead to inflammasome activation (Figure 3.13C). MDP alone was only able to activate a very low number of the cells, whereas cells stimulated with LPS and ATP had a significant incidence (>30%) of ASC specks.

(A)

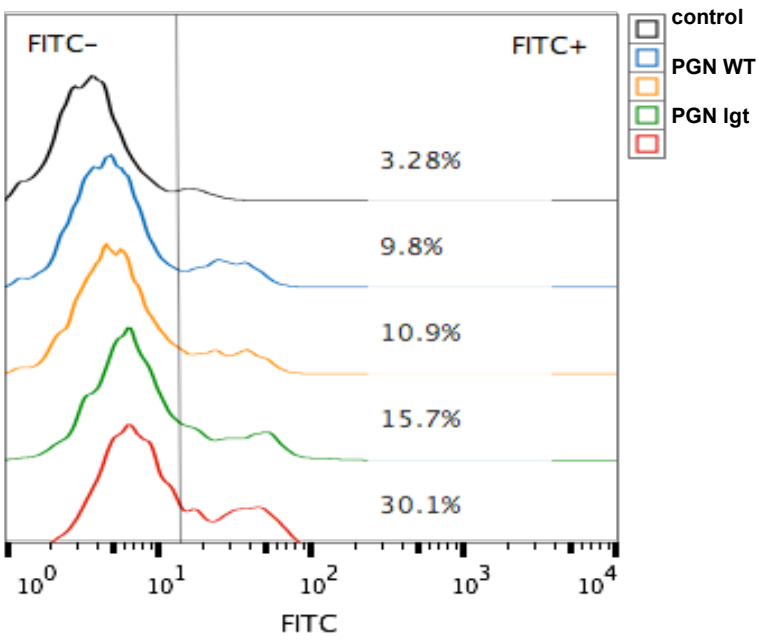

Figure 3.13. *C. difficile* PGN does not activate the inflammasome.

dTHP-1 cells were stimulated with 1µg/ml *C. diffiicile* WT or *C. difficile lgt* PGN, MDP or 10ng/ml *E. coli* LPS and ATP (30nM). (A) 4h post-stimulation, active caspase-1 was detected using the Flica assay and analysed by flow cytometry. Representative histogram from two independent experiments is shown.

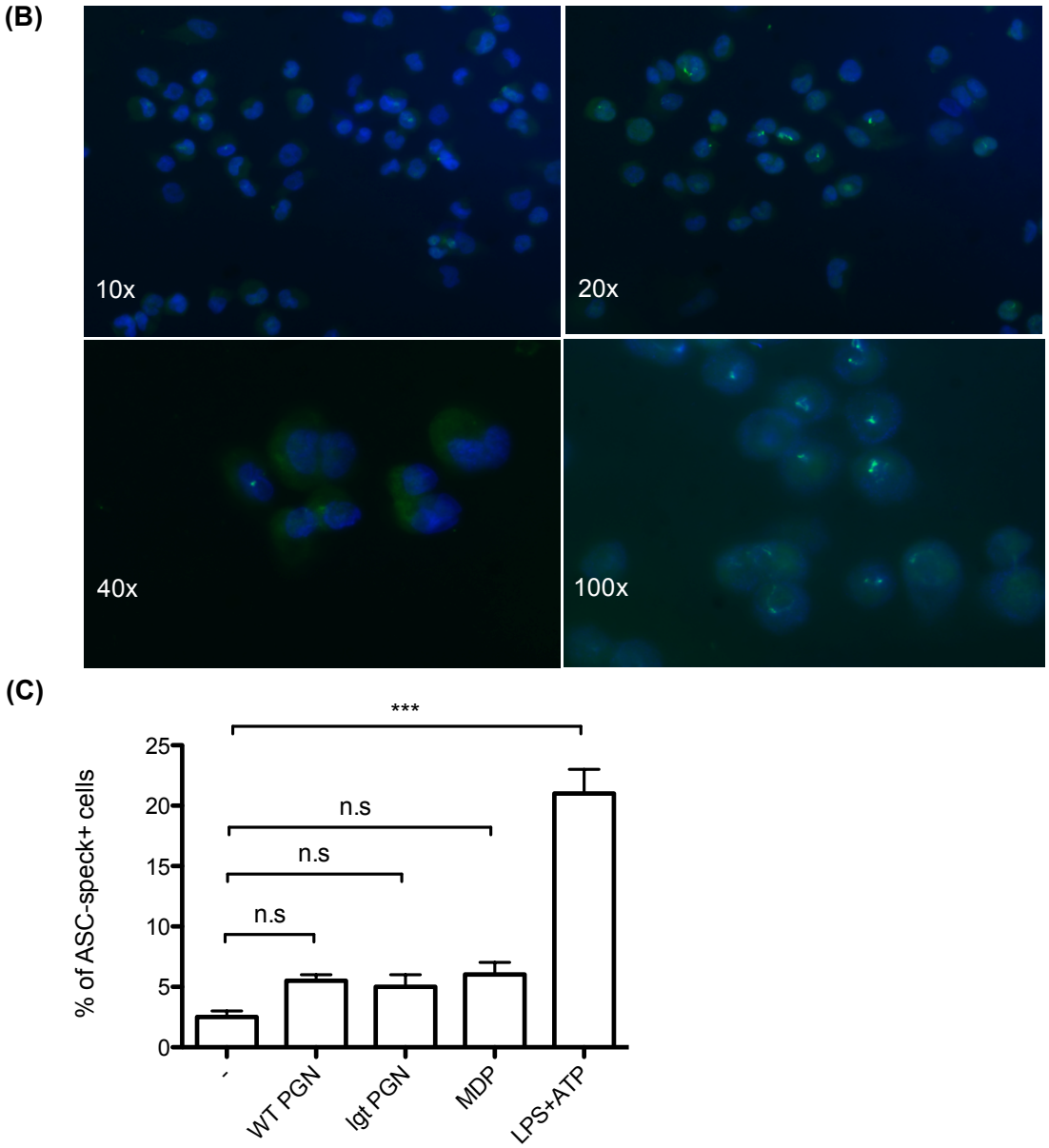

Figure 3.13. *C. difficile* PGN does not activate the inflammasome (cont.).

dTHP-1 cells were stimulated with 1µg/ml *C. diffiicile* WT or *C. difficile lgt* PGN, MDP or 10ng/ml *E. coli* LPS and ATP (30nM). (B) 4h post-stimulation, oligomerised ASC was detected in TDM monolayers grown on coverslips using a fluorocrome-conjugated anti-ASC antibody and visualised by laser microscopy (Pictures taken at 10x, 20x, 40x and 100x magnification). (C) Number of ASC "specks" from ten fields chosen at random presented as percentage of total cells. Values are means ± SEM from two independent experiments. Statistical analysis by Mann-Whitney test (***P<0.001).

3.3 Discussion

Ultasonication is routinely used to disperse aggregates that form during PGN isolation (295). The strong shearing forces employed are capable of breaking covalent bonds, causing the release of PGN fragments and altering the composition of the preparation into a mix of intact PGN and smaller soluble polymers. The observed dose-dependent increase in TLR2 activation following sonication of PGN (Figure 3.1) can be attributed to the detachment of such complexes from the PGN. Interestingly, while both *C. difficile* and *S. aureus* PGN induced comparable TLR2 activation at 6h, the former was a far more potent inducer of IL-8 at 24h (Figure 3.2). IL-8 induction was nearly maximal at a 1μg/ml dose of *C. difficile*, while the dose-dependent increase in TLR2 activation by S. aureus was similar to that reported elsewhere (28). Since we demonstrated that lipoproteins anchored to the PGN are responsible for TLR2 activation (Figure 3.6), this difference may be due to the lipoprotein content of the two PGNs; nearly 50 lipoproteins are predicted by the genome sequence of *S. aureus* (304) compared to 85 for *C. difficile* (138). Differences also likely exist in the processing of PGN and release of its various moieties within the cell. These stem from modifications in the sugar backbone of PGN, mainly N-deacetylation or O-acetylation, which affect its resistance to-degrading enzymes, such as lysozyme, and are important for bacterial survival. Peltier *et al.* (156) found that 93% of GlcNAc in the *C. difficile* PGN is deacetylated, conferring the pathogen resistance to lysozyme. Increased lysozyme resistance is an important virulence factor for numerous Gram-positive bacteria, including *Listeria monocytogenes* and *Streptococcus pneumoniae* which are N-deacetylated whilst *S. aureus* which is O-acetylated (305). Resistance to lysozyme-induced degradation is likely to lead to delayed release of TLR2 activating ligands from *C. difficile*.

In dTHP-1 cells, PGN from *C. difficile* was able to induce NF-κB activation at levels comparable to those of *S. aureus* PGN (Figure 3.5). Although 10μg/ml of PGN was

able to drive NF-κB activation equal to that of LPS stimulation, empirically, LPS would be expected to induce more NF-κB as it is the foremost inflammatory trigger. This may imply that our reporter construct signalling has reached saturation and therefore, quantitative comparisons are not wholly appropriate in this experimental system. Nevertheless, both WT and *lgt C. difficile* PGN were capable of NF-κB induction in dTHP-1 cells (Figure 3.7). The lack of TLR2 signalling in *lgt* PGN-treated cells would account for some loss in NF-κB activation and the time frame of NF-κB activation would indicate PGN recognition occurs later than TLR2-medated lipoprotein signalling. This is most likely due to the requirement for PGN internalisation and hydrolysis prior to its recognition by NOD1 and NOD2 (286). Nevertheless, *lgt C. difficile* PGN was also able to induce NOD-dependent NF-κB induction in non-phagocytic HEK293 cells (Figure 3.8). It is worth noting that our PGN preparation was free of TLR4 or TLR5 agonists (Figure 3.3) whose presence has been reported in commercially available PGN preparations (306) and would lead to immune cell activation.

Another unique feature of *C. difficile* PGN is the presence of DAP moieties, commonly found in Gram-negative organisms, with the exception of Gram-positive bacilli (289). *C. difficile* contains both DAP and MDP motifs which are the respective ligands of NOD1 and NOD2. As anticipated, both NOD1 and NOD2 were able to recognise *C. difficile* and induce NF-κB activation in NLR-transfected cells (Figure 3.8). MDP sensing by NOD2 has been well-documented (40,307) in both Gram-positive and Gram-negative pathogenic bacteria, such as *Streptococcus pneumonia* (101), *Mycobacterium tuberculosis* (308), *S. aureus* (79), *Salmonella* serotype typhimurium (79) and *Bacillus anthracis* (95). The role of NOD1 in PGN recognition was first reported by Inohara *et al.* (46) studying the PGN of *Bacillus subtilis*, followed by reports on the PGN of *B. anthracis* (286) and *Listeria monocytogenes* (309). In the case of non-invasive pathogens, such as *Helicobacter pylori,* NOD1 can recognise PGN delivered to the

cytosol (169). The moderate levels of NF-κB induction may indicate the requirement for PGN degradation by lysozyme, or *ex-vivo* treatment with hydrolases, for the generation of NOD ligands as others have reported (172). The role of NOD1 in immune sensing of *C. difficile* was established in a recent study where NOD1- but not NOD2-deficient mice exhibit a compromised immune response to *C. difficile* infection (77), however the specific ligands were not identified.

The presence of DAP-type PGN on all Clostridia bacilli strongly indicates that they too may possess NOD1-stimulating activity. Therefore, the importance of NOD-mediated recognition stretches beyond the innate response to *C. difficile*, since NOD1 regulates the Clostridiales population in the gut and NOD1-mediated detection is essential for the development of adaptive immunity and the maintenance of intestinal homeostasis (310). Also, NOD1-mediated sensing of PGN from the microbiota in mice, but not NOD2- or TLR4-mediated signalling, is important for the systemic priming of the innate immune response against pathogens (311). The frequency of several bacterial genera of Clostridia (312) NOD2 activation by commensals (including Clostridia), plays multiple roles in the maintenance of intestinal immune equilibrium. Moieties released from commensal bacteria can offer protection from induced colitis in mouse models in a NOD2-dependent manner (313). Petnicki-Ocwieja *et al.* (86) noted that lack of NOD2 resulted in an increased load of certain commensal bacteria in the terminal ileum and defects in bacterial clearance from ileal crypts of the intestine has been reported in Crohn's disease patients with NOD2 polymorphisms (314).

Similar to NF-κB activation, the pro-inflammatory cytokine response to PGN was mediated by both NOD1 and NOD2 in primary macrophages (Figure 3.12). A decrease in the TNFα response was observed following knockdown of both NOD proteins, but only became statistically significant in NOD2-knockdown cells. This discrepancy can be

attributed to factors that lie both in experimental limitations and the complex interplay of NOD signalling. Receptor over-expression in HEK293 cells is a widely adopted method for ligand-receptor studies, however, excessive NOD protein expression may lower their activation threshold (315), (Personal communication). While the use of HEK293 cells offers a reductive model for signalling studies, it cannot account for the plurality of functions of receptors such as these. Since a certain degree of redundancy exists between the NODs (85); it is possible that NOD2 signalling has compensated for the lack of NOD1 activity. And despite both proteins sharing downstream effectors that control TNFα expression, only a mutation in NOD2 has been linked to the development of disease (41). However, the literature remains conflicting over the outcomes of NOD activation in part due to the differences between human and mouse models (316). Identifying those parts of the PGN polymer involved in NOD1 and NOD2 activation and establishing their individual binding affinity, as others have done for the of *E. coli* (317), would shed more light on the role of each receptor.

To this day, no reports of PGN-induced inflammasome activation exist. Not surprisingly, PGN from *C. difficile* did not activate the inflammasome (Figure 3.13). Despite the existence of limited evidence of inflammasome activation by MDP alone, the majority of the literature does not support that notion and neither did our work. Similarly for lipoproteins, although in study demonstrating inflammasome activation by the lipoproteins of *S. aureus*, secreted lipoproteins rather than those attached to the PGN scaffold were used and that may explain the different outcome (293).

Chapter 4

Innate immune response to *Clostridium difficile* PGN

4.1 Introduction

In Chapter 3, we described the interaction of *C. difficile* PGN with the NOD receptors and its effect on NF-κB signaling, which controls the expression of key secreted pro-inflammatory mediators of innate immunity. We therefore wanted to investigate the immunogenic properties of *C. difficile* PGN, similar to studies of PGN from *B. anthracis* (318) and *S. aureus* (62). We set out to analyse the cytokine response to WT and *lgt C. difficile* PGN and identify their source amongst peripheral blood cells. Besides inducing an acute response from immune cells, bacterial compounds can inform their functional fate and instruct their maturation. MDP moieties of PGN have been shown to act cooperatively with low doses of LPS to induce the maturation of DCs (69). We hypothesised that the PGN of *C. difficile* may exhibit similar properties and proceeded to analyse the expression of co-stimulatory molecules and receptors in primary monocytes and macrophages.

With the intestinal environment being littered with MAMPs, including PGN, intestinal immune cells are constantly presented with a concoction of antigens. Following the discovery of TLRs and NLRs, a lot of effort has gone into identifying how these two receptor families orchestrate host defence. Numerous studies have shown that NOD1, NOD2 and TLR signalling pathways intersect at multiple levels in cellular systems (319). Using ligands to co-activate NODs and TLRs simultaneously or sequentially, researchers have discovered that NOD-mediated signalling can modulate TLR-induced cytokine production and cell maturation. NOD2 activation acts synergistically to enhance the TNFα response to TLR2 and TLR4 ligands (69). And vice versa, lipoprotein-mediated activation of TLR2 can boost the effects of NOD2 stimulation by PGN (320). This synergistic action is important for the production of both pro- and anti-inflammatory cytokines. In a recent study, NOD2 was shown to directly interact with a transcriptional controller of the IL-10 promoter (95). Furthermore, a joint role for TLRs

and NOD2 in triggering production of IL-10 has been described (53). Since NOD-mediated modulation of TLR signalling can greatly affect the outcome of infection, we set out to investigate how the monocytic immune response to various pathogen-derived ligands is affected in the presence of *C. difficile* PGN,

Beyond the acute response, stimulation with bacterial compounds can alter the functional fate of innate immune cells. This is done through the desensitisation or enhancement of the immune response of naive cells via the processes of tolerance or trained immunity respectively. LPS-induced tolerance has been known for decades and describes the diminished cytokine response to LPS upon secondary stimulation (321). More recently, the role of NOD2 in the development of LPS tolerance by monocyte-derived macrophages (MDMs) was described (81). Trained immunity, on the other hand, refers to an enhanced cytokine response after a second encounter with a pathogen. This enhancement offers a protective effect to monocytes and macrophages that are thought to exhibit memory characteristics. Amongst the bacterial ligands known to enhance the cytokine response of MDMs were MDP and iE-DAP (322). As no reports exist on the effect of chronic PGN stimulation, we examined the effect of prolonged *C. difficile* PGN treatment on the macrophage response to LPS.

In this chapter we aimed to:

1. Characterise the immune cell phenotype and secreted mediators of the innate immune response to *C. difficile* PGN.

2. Investigate the cross-talk between *C. difficile* PGN- and TLR-induced signaling.

4.2 Results

4.2.1 Cytokine response to *C. difficile* PGN

NOD receptors are known to mediate the cytokine response to PGN moieties in APCs (see Chapter 3). Here, the ability of *C. difficile* PGN to trigger the production of cytokines in peripheral blood mononuclear cells (PBMCs) was investigated. While PBMCs may not closely mirror mucosal immune cells (323), they are an accessible source of TLR- and NLR-expressing cells. Freshly isolated PBMCs were treated with 1µg/ml PGN from *C. difficile* or *S. aureus* and the production of secreted chemokines and cytokines assessed. The two main pro-inflammatory mediators of the monocytic response to *S. aureus* PGN were analysed: IL-8 and TNFα (324). Both are predominantly mediated by TLR2 activation (26),(325). Treatment with *C. difficile* PGN induced significant levels of secreted IL-8 (P=0.086), ~ 6900pg/ml after 6h and 9800pg/ml after 24h (Figure 4.1). Comparatively, PGN from *S. aureus* induced lower levels of IL-8 (~ 4000pg/ml); however the difference was not statistically significant. TNFα production only became significant after 24h of treatment (P=0.0014) reaching ~ 9000pg/ml. Neither PGN was able to elicit significant levels of IL-1β, while only PGN from *S. aureus* induced secretion of IL-10.

We then wanted to identify the source of this cytokine response. Collected PBMCs were sorted into CD14-/lymphocytic and CD14+/monocytic fractions. Neutrophils from whole blood were also included in the study. The cells were stimulated with PGN at 0.1 and 1µg/ml for 24h before assessing IL-8 and TNFα levels in the supernatant. As Figure 4.2 shows, stimulation with 0.1µg/ml induced only low levels of IL-8 in PBMCs (~ 800pg/ml) (Figure 4.2A). The majority of IL-8 was produced by monocytes (~ 9000pg/ml) (Figure 4.2B), while modest amounts were secreted by neutrophils (~ 1200pg/ml) (Figure 4.2D) and only low amounts by the CD14- fraction (granulocytes, T-

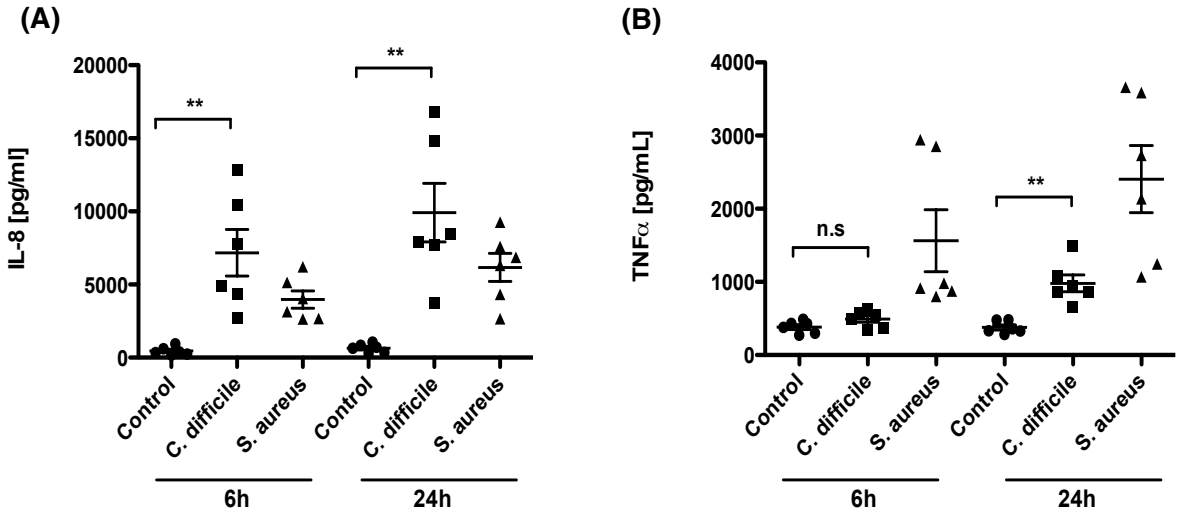

Figure 4.1. *C. difficile* PGN elicits a cytokine response in peripheral blood mononuclear cells (PBMCs).

5×10^5 peripheral blood mononuclear cells (PBMCs) were stimulated with 1µg/ml *C. difficile* or *S. aureus* PGN for 6 and 24h. (A) IL-8 chemokine and (B) TNFα, (C) IL-1β and IL-10 cytokine levels were measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (**P<0.01).

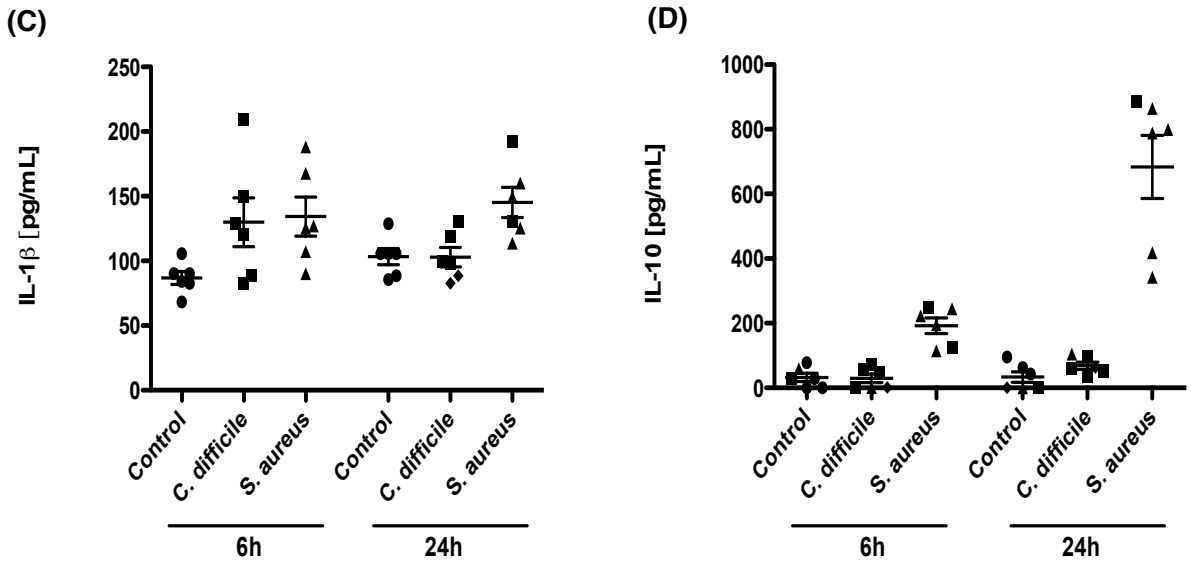

Figure 4.1. *C. difficile* PGN elicits a cytokine response in peripheral blood mononuclear cells (PBMCs) (continued).

5×10^5 peripheral blood mononuclear cells (PBMCs) were stimulated with 1µg/ml *C. difficile* or *S. aureus* PGN for 6 and 24h. (A) IL-8 chemokine and (B) TNFa, (C) IL-1β and IL-10 cytokine levels were measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (**P<0.01).

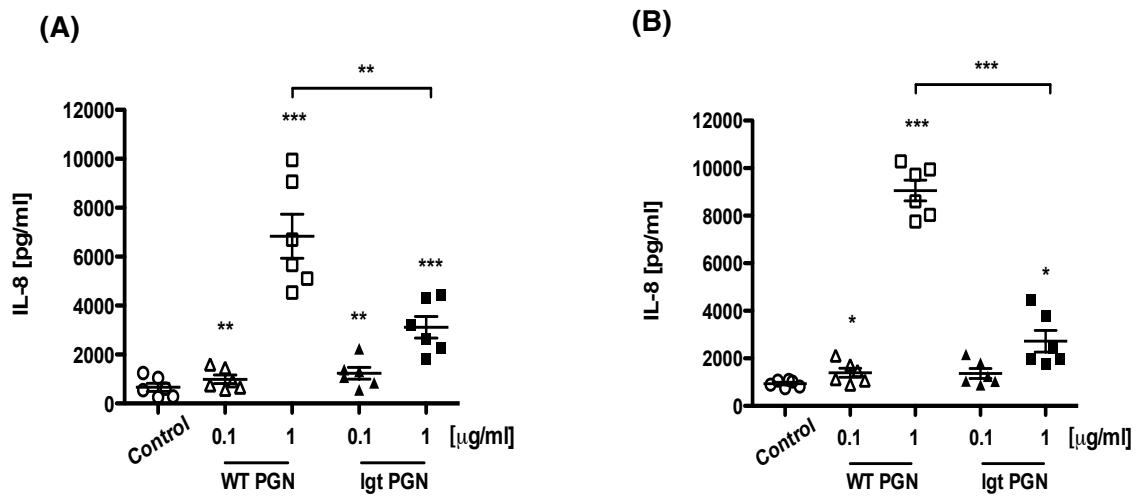

Figure 4.2. CD14+ monocytes are the major source of *C. difficile* PGN-mediated IL-8 response.

(A) PBMCs, (B) CD14+ monocytic or (C) CD14- PBMC fractions and (D) neutrophils were stimulated with 0.1 and 1μg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation, IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

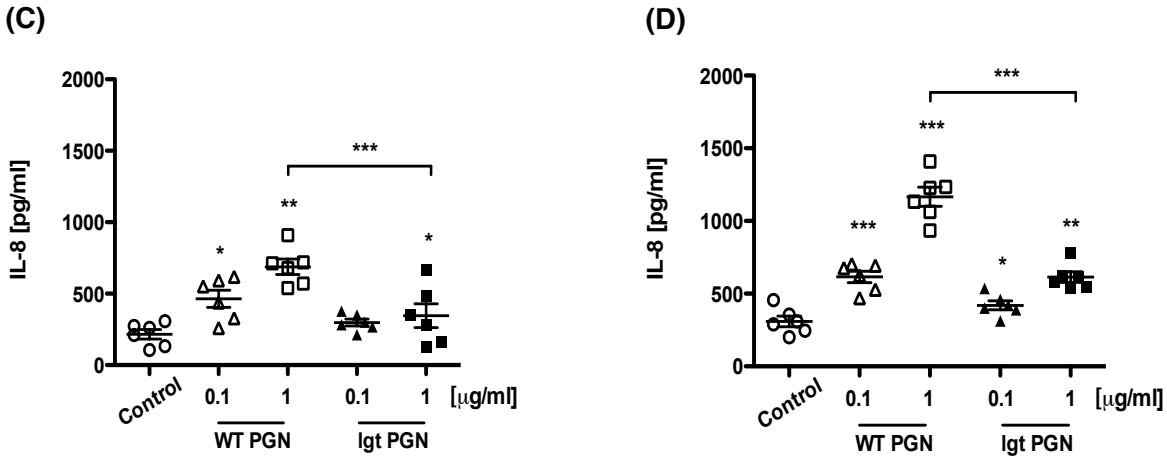

Figure 4.2. CD14+ monocytes are the major source of *C. difficile* PGN-mediated IL-8 response (continued).

(A) PBMCs, (B) CD14+ monocytic or (C) CD14- PBMC fractions and (D) neutrophils were stimulated with 0.1 and 1μg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation, IL-8 was measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

cells) (Figure 4.2C). To distinguish the contribution of PGN-associated lipoproteins from that of PGN, cells were also treated with 0.1 and 1µg/ml *lgt C. difficile* PGN. At a concentration of 0.1µg/ml the immune response was weak and no significant difference was observed between WT and *lgt* PGN-induced IL-8 or TNFα. Treatment with 1µg/ml *lgt C. difficile* PGN induced lower levels of IL-8 across all cell fractions in comparison to WT PGN (Figure 4.2). The difference between the two PGN preparations was more pronounced in CD14+/monocytes where WT PGN induced ~ 9000pg/ml and *lgt* PGN ~ 3000pg/ml (Figure 4.2B).

Stimulation of PBMCs with 0.1 and 1µg/ml PGN led to the secretion of ~ 400 and 4900pg/ml TNFα respectively (Figure 4.3A), with monocytes being the main source of the cytokine and producing ~ 7500pg/ml (Figure 4.3B). In contrast to IL-8, there was greater contribution from the CD14- fraction (~ 1200pg/ml) (Figure 4.3C) and none from neutrophils (Figure 4.3D). While PBMCs secreted less TNFα when treated with *lgt* compared to WT *C. difficile* PGN (~ 3200 and 4900pg/ml respectively), the difference was not statistically significant (Figure 4.3A). However, when CD14+/monocytes and CD14-/lymphocytes were analysed, the difference between the two treatments was significant (P=0.0124) (Figure 4.3B). No significant difference in TNFα induction was detected in the remaining cell fractions (Figure 4.3C and D). Finally, PGN from *C. difficile* was able to induce both IL-6 and IL-10 in monocytes with median ~ 640 and 520pg/ml respectively, but only low levels of IL-12 (Figure 4.4). Treatment with 1µg/ml lipoprotein-negative *lgt* PGN led to a significant reduction in IL-6 production (P=0.024). Notably, IL-10 levels were comparable between cells treated with WT and *lgt C. difficile* PGN as no statistically significant difference was detected. Taking into account their central role in cytokine-mediated immunity to PGN, monocytes were used to conduct further experiments.

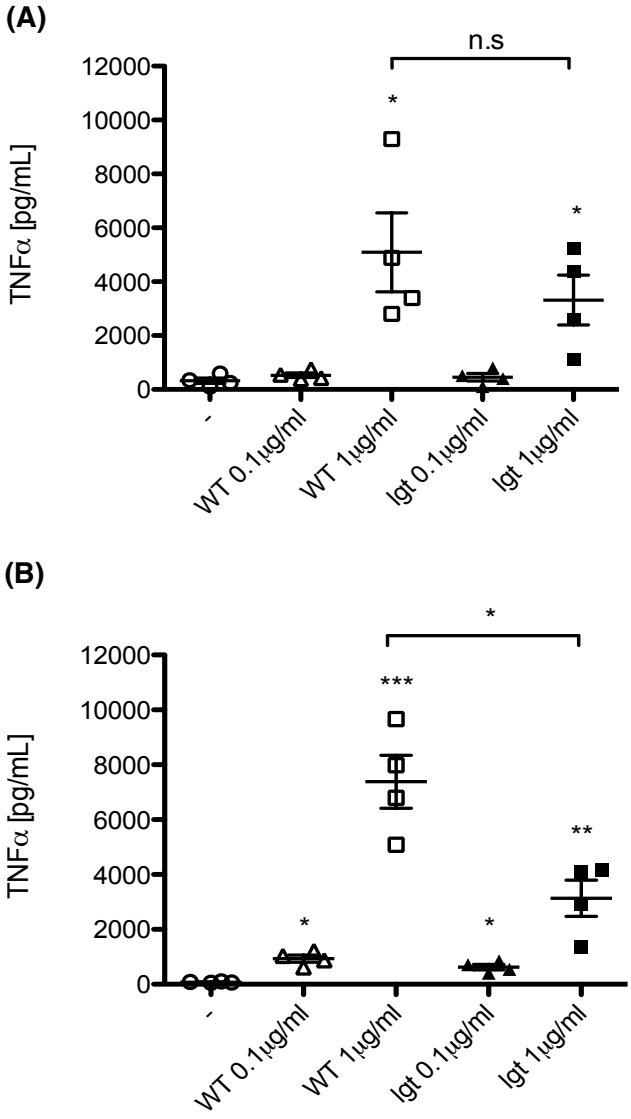

Figure 4.3. CD14+ monocytes are the major source of *C. difficile* PGN-mediated TNFα response.

(A) PBMCs, (B) CD14+ monocytic or (C) CD14- PBMC fractions and (D) neutrophils were stimulated with 0.1 and 1μg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation, TNFα was measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

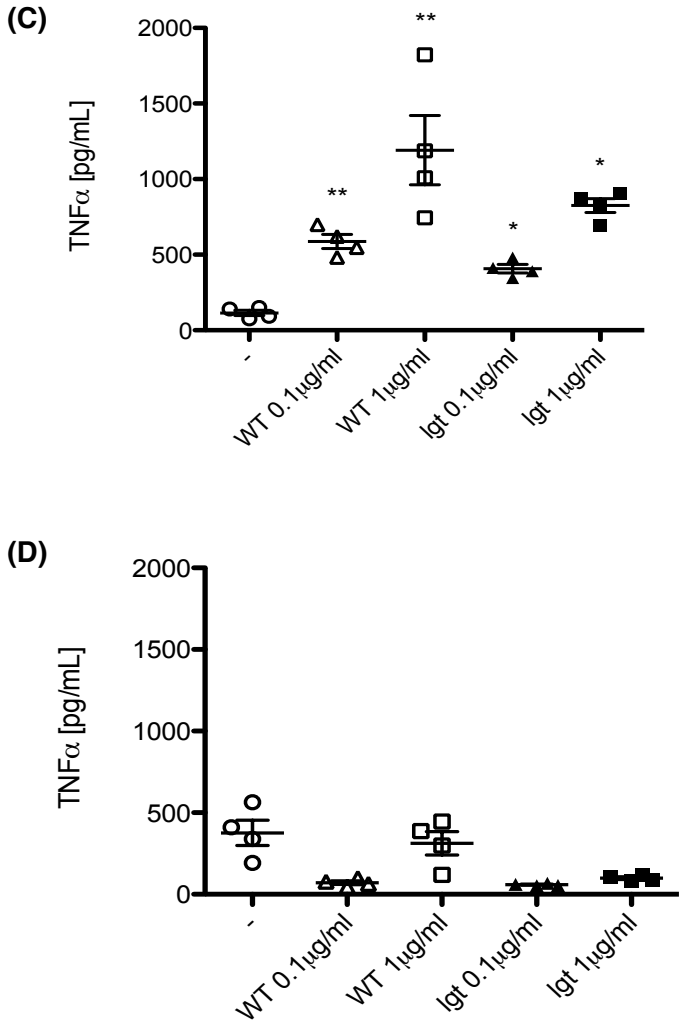

Figure 4.3. CD14+ monocytes are the major source of *C. difficile* PGN-mediated TNFα response (continued).

(A) PBMCs, (B) CD14+ monocytic or (C) CD14- PBMC fractions and (D) neutrophils were stimulated with 0.1 and 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation, TNFα was measured in the supernatant by ELISA. Values represent mean ± SEM from six individuals analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

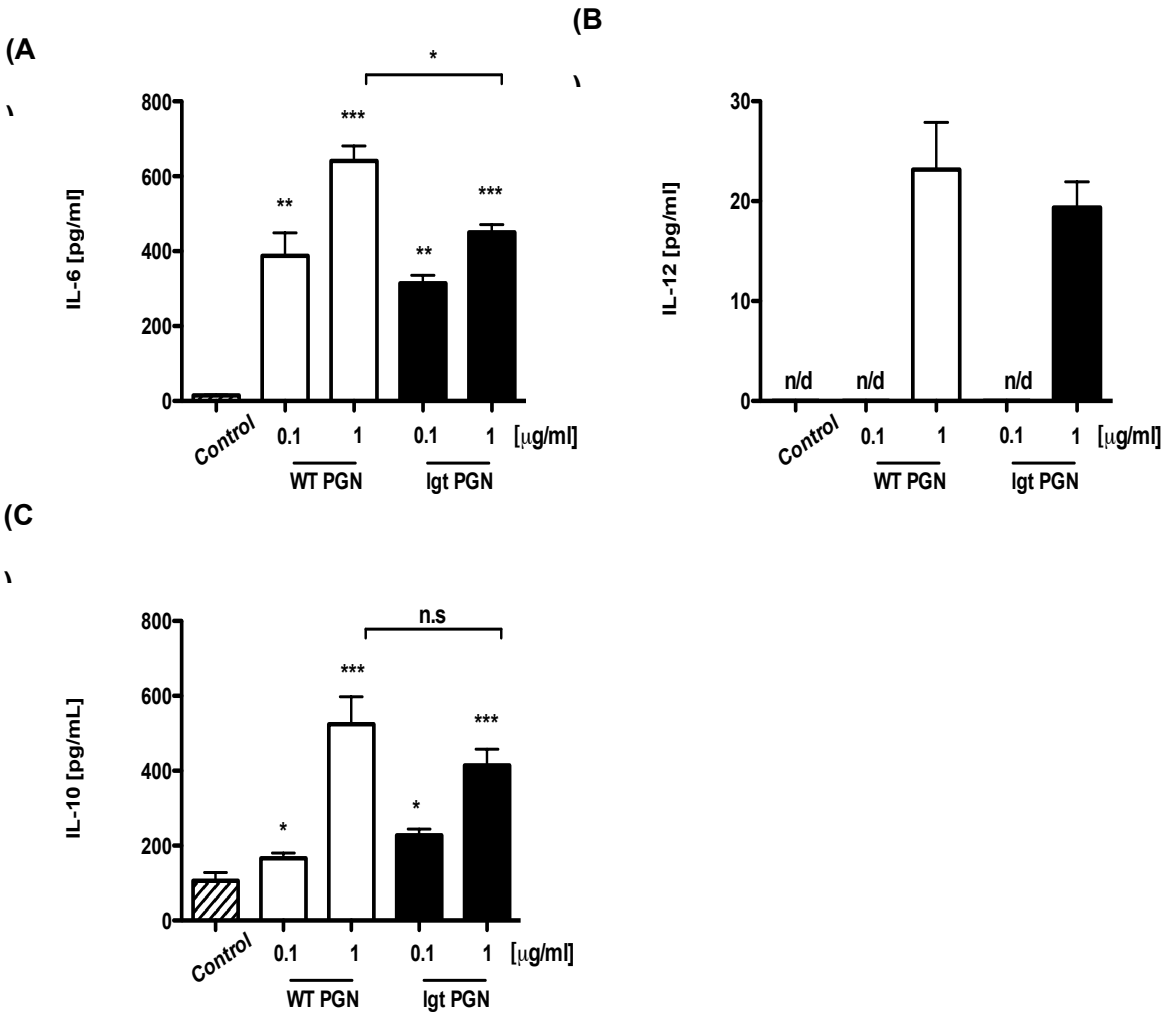

Figure 4.4. Cytokine response to *C. difficile* PGN in human monocytes.

CD14+ monocytes were stimulated with 0.1 and 1μg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation, IL-6, IL-10 and IL-12 cytokines were measured in the supernatant by ELISA. Values represent mean ± SEM from four individuals analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

4.2.2 Synergistic role of *C. difficile* PGN-mediated NOD activation in TLR signalling

Cytokine production is only one aspect of the multifaceted immune response to bacterial antigens. Simultaneously, innate immune cells up-regulate the expression of PRRs in order to enhance their surveillance capabilities. Surface molecules involved in cross-activation of other immune cells also become up-regulated as monocytes differentiate to mature APCs. Wanting to assess the capacity of *C. difficile* PGN to induce the maturation of naïve immune cells, we analysed the expression of the MHC Class II receptor HLA-DR, which becomes up-regulated in monocytes undergoing differentiation. We also examined the co-stimulatory molecules CD80 and CD86, found in mature macrophages and CD40, another co-stimulatory protein found on APCs. Treatment with PGN from WT *C. difficile* led to significant up-regulation of CD86 (P=0.03), but had no effect on any of the other molecules (Figure 4.5). The increase in CD86 expression was lipoprotein-mediated, since treatment with *lgt* PGN did not lead to significant change in marker expression. We also analysed the expression of TLR2 and TLR4, two of the key receptors in the sensing of bacterial cell wall components. Treatment with PGN from WT *C. difficile* led to significant up-regulation of TLR4 (P=0.03) (Figure 4.6), again mediated by lipoproteins rather than the PGN itself, as WT and *lgt* PGN preparations showed no difference.

As *C. difficile* PGN did not have an effect on its own, we were eager to examine its ability to modulate LPS-induced monocytic maturation. Previously, others have reported the capacity of MDP to modulate LPS-mediated surface marker expression in DCs(69). As expected, stimulation of monocytes with LPS led to significant up-regulation of HLA-DR (P=0.026) as well as the co-stimulatory factors CD40 (P=0.04) and CD80 (P=0.032) (Figure 4.7). However, we found no evidence that co-stimulation with *lgt* PGN had a significant impact on the LPS-induced expression of these proteins.

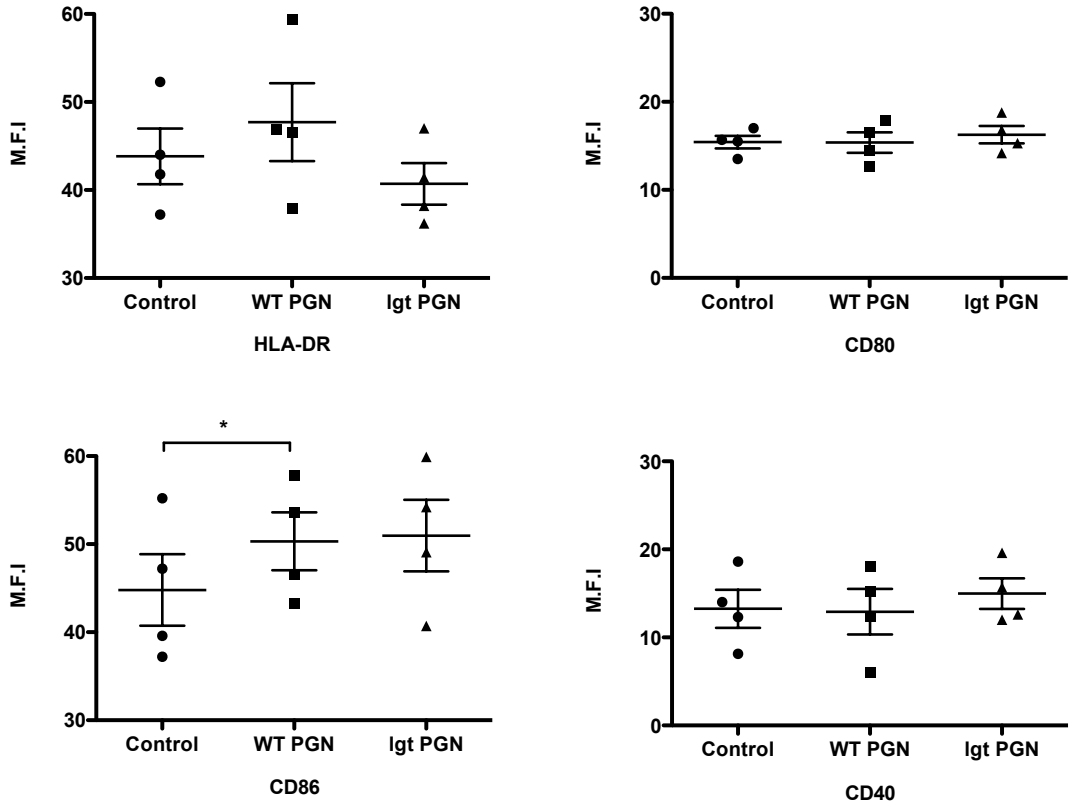

Figure 4.5. MHC class II and co-stimulatory molecule expression in monocytes in response to *C. difficile* PGN is lipoprotein-mediated.

2.5×10^5 CD14+ monocytes were stimulated with 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation the expression of MHC class II receptor HLA-DR and co-stimulatory proteins CD80, CD86 and CD40 was analysed by flow cytometry. Data is presented as M.F.I and values represent mean ± SEM from four individuals. Statistical analysis by paired t-test (*P<0.03).

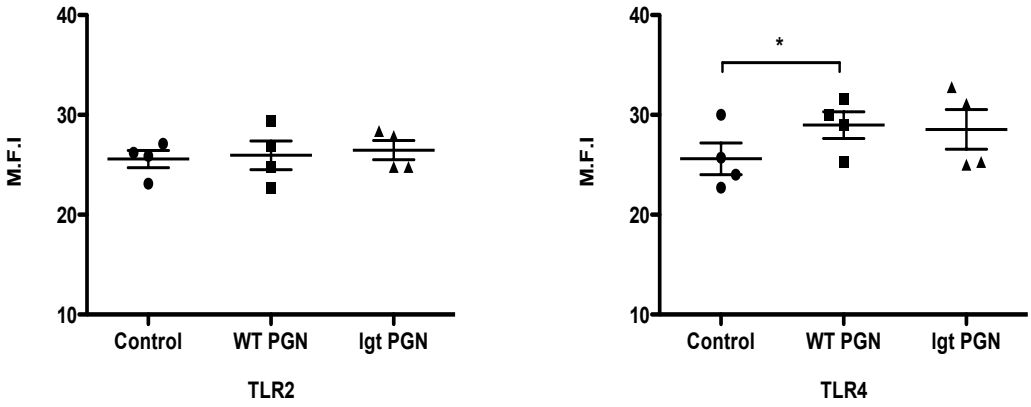

Figure 4.6. TLR4 up-regulation in the monocytic response to *C. difficile* PGN is lipoprotein-mediated.

2.5×10^5 CD14+ monocytes were stimulated with 1µg/ml *C. difficile* WT or *C. difficile lgt* PGN. 24h post-stimulation TLR2 and TLR4 expression was assessed by flow cytometry. Data presented as M.F.I and values represent mean ± SEM from four individuals. Statistical analysis by paired t-test (*P<0.05).

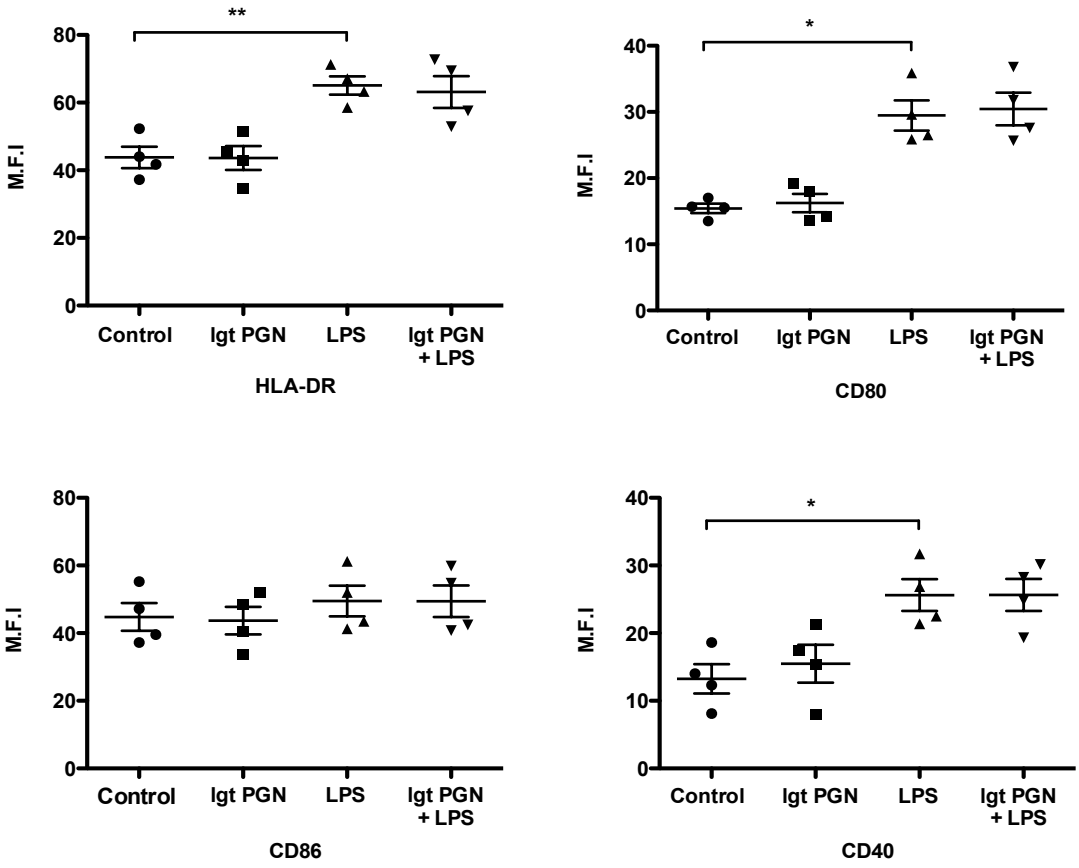

Figure 4.7. Potential modulatory effect of *C. difficile* PGN on monocytic activation.

2.5×10^5 CD14+ monocytes were stimulated with 1µg/ml *C. difficile lgt* PGN, 10ng/ml LPS or both. 24h post-stimulation, expression of co-stimulatory proteins was analysed by flow cytometry. Data presented as M.F.I and values represent mean ± SEM from four individuals. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

Likewise, TLR2 expression was significantly higher (P=0.04) following treatment with LPS, yet was not modulated in the presence of *C. difficile* PGN (Figure 4.8). While we did not observe any affect of *C. difficile* PGN on TLR4-mediated surface molecule expression, we went on to analyse the cytokine response of monocytes co-stimulated with PGN and LPS based on previous studies underlining the synergistic role of NOD receptors in the TLR-driven cytokine production of monocytes and lymphocytes(326). *C. difficile* PGN appeared to enhance the TNFα (P=0.044) and IL-6 (P=0.046), a cytokine closely related to TNFα, response to LPS (Figure 4.9). However, no significant change in IL-10 secretion was observed. These data suggested that, although the expression of surface markers was unchanged, PGN from *C. difficile* can modulate TLR-mediated pro-inflammatory signaling.

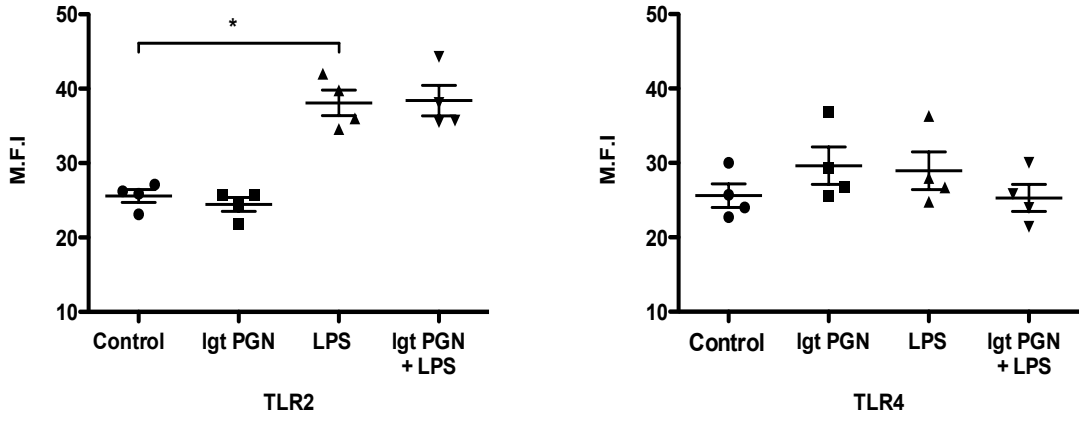

Figure 4.8. Co-stimulation with *C. difficile* PGN does not modulate TLR expression.

2.5×10^5 CD14+ monocytes were stimulated with 1µg/ml *C. difficile lgt* PGN, 10ng/ml LPS or both. 24h post-stimulation, expression of TLR2 and TLR 4 was analysed by flow cytometry. Data is presented as M.F.I and values represent mean ± SEM from four individuals. Statistical analysis by paired t-test (*P<0.05).

We then went on to test the capacity of *C. difficile* PGN to modulate the cytokine response to other TLR agonists. Interestingly, the TNFα cytokine response to all TLR agonists was enhanced in the presence of *C. difficile* PGN (TLR2; P=0.024, TLR3; P=0.035, TLR5; P=0,039) (Figure 4.10). There was also a significant increase in the expression of IL-6 following co-stimulation with *S. aureus* PGN (TLR2; P=0.012) and flagellin (TLR5; P=0.45), but not polyI:C (TLR3; P=0.263). *C. difficile* PGN significantly enhanced the IL-10 response to polyI:C (TLR3; P=0.042) and flagellin (TLR5; P=0.034), but not TLR2; despite an increase in IL-10 production from ~400 to 550pg/ml.

To further assess the contribution of *C. difficile* PGN to TLR-mediated signaling and look for evidence of synergy, the ELISA results were expressed as ratio of the median value induced by *lgt* PGN and the corresponding TLR ligand combined *versus* the sum value of their independent effect, as previously described (327). Our analysis indicates that co-stimulation with *lgt* PGN had a synergistic effect in LPS-induced TLR4 signalling for all three cytokines since the value of the ratio was equal or greater to 1.5 (Figure 4.11).

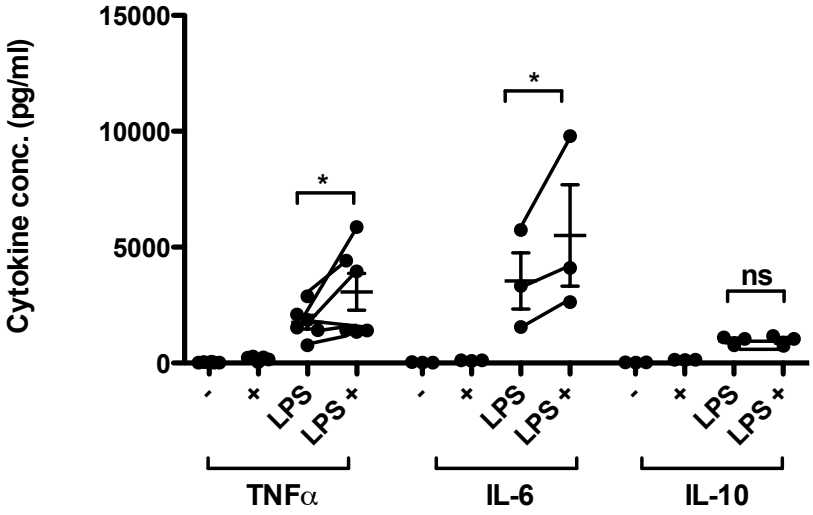

Figure 4.9. *C. difficile* PGN plays a synergistic role in TLR4-mediated cytokine induction in human monocytes.

5×10^5 CD14+ monocytes were stimulated with 10ng/ml *E. coli* LPS alone or in the presence of 1µg/ml *C. difficile lgt* PGN (+) for 24h. TNFα, IL-6 and IL-10 cytokine levels were measured in the supernatant by ELISA. Values represent mean ± SEM (n>=3) analysed in duplicate. Statistical analysis by paired t-test (*P<0.05).

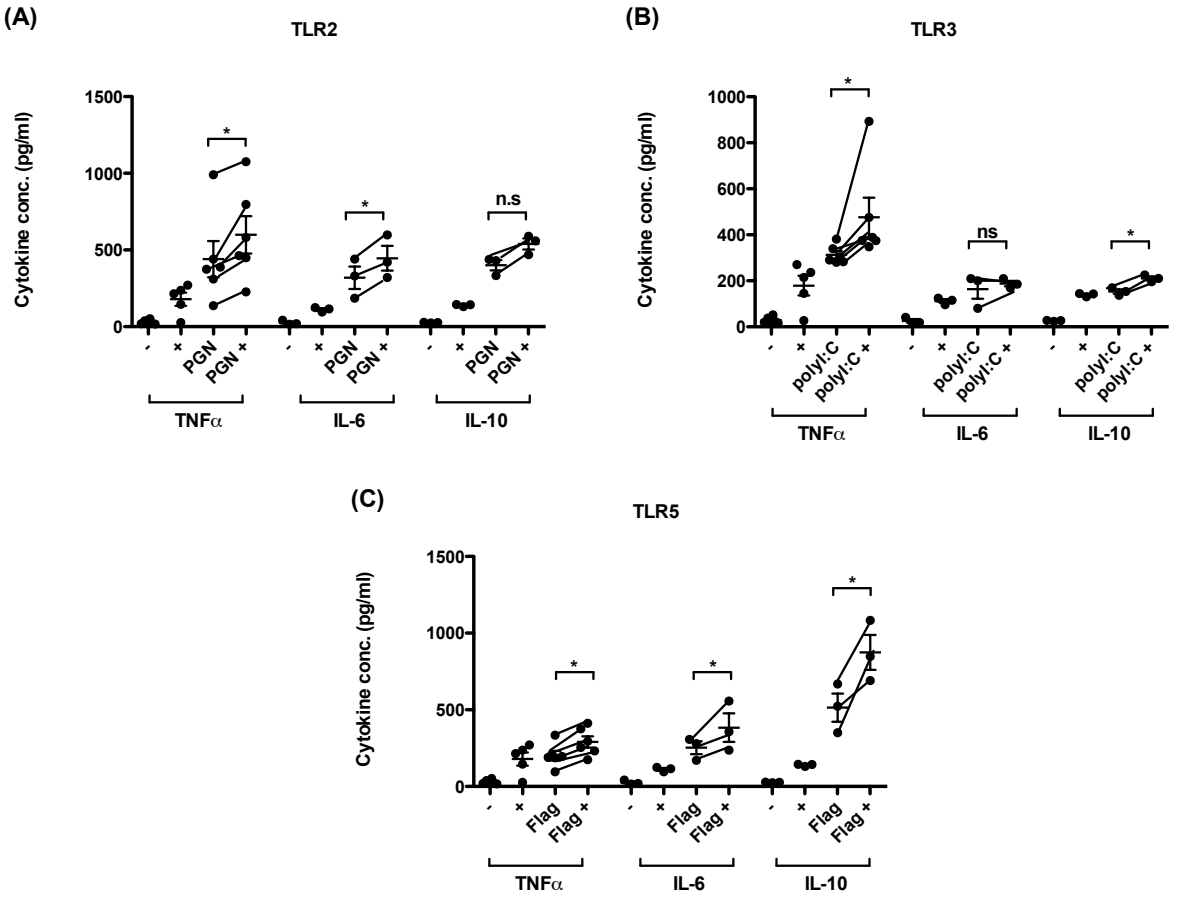

Figure 4.10. *C. difficile* PGN plays a synergistic role in TLR-mediated cytokine induction in human monocytes.

5×10^5 CD14+ monocytes were stimulated with (A) 1µg/ml PGN from *S. aureus* (TLR2), (B) 50ng/ml polyI:C, (TLR3), (C) 5ng/ml *Salmonella* typhimurium flagellin (TLR5) alone or in the presence of 1µg/ml *C. difficile lgt* PGN (+) for 24h. TNFα, IL-6 and IL-10 cytokine levels were measured in the supernatant by ELISA. Values represent mean ± SEM (n>=3) analysed in duplicate. Statistical analysis by paired t-test (*P<0.05).

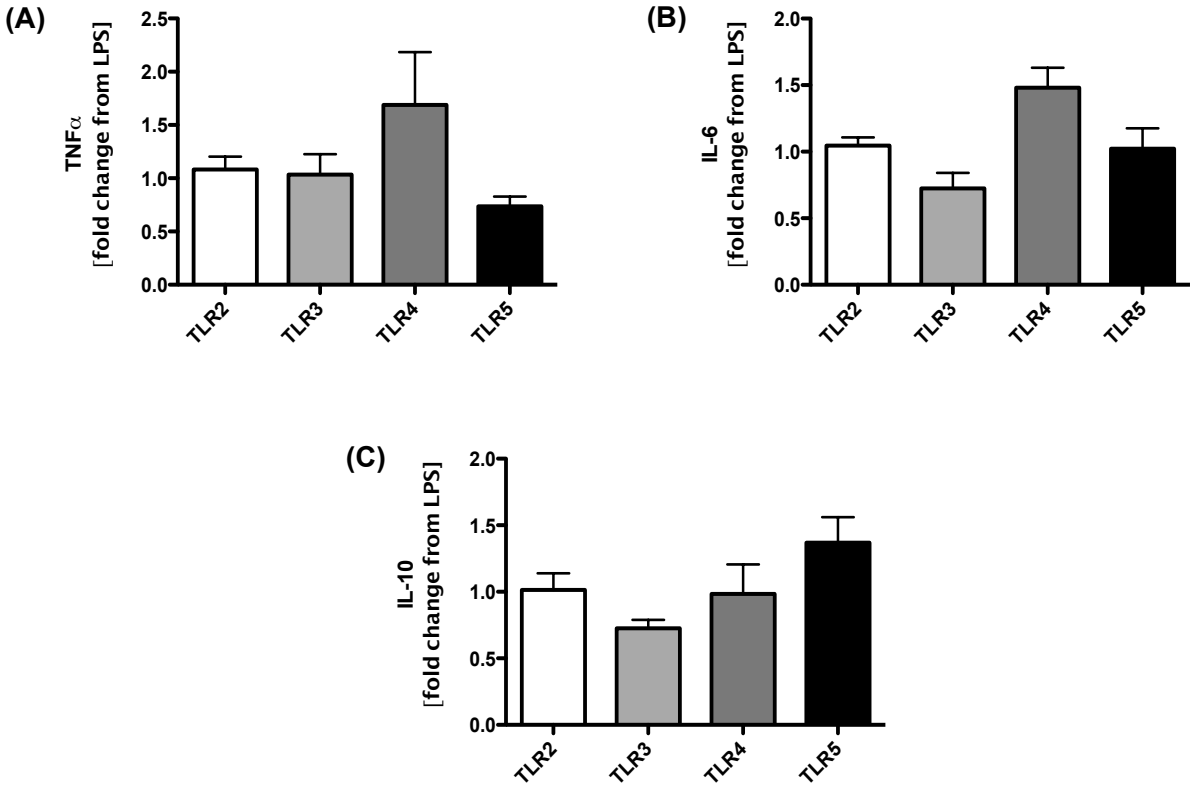

Figure 4.11. *C. difficile* PGN plays a synergistic role in TLR-mediated cytokine induction in human monocytes.

5×10^5 CD14+ monocytes were stimulated with (A) 1µg/ml PGN from *S. aureus* (TLR2), (B) 50ng/ml polyI:C (TLR3), (C) 50ng/ml *Salmonella* typhimurium flagellin (TLR5) alone or in the presence of 1µg/ml *C. difficile lgt* PGN for 24h. TNFα, IL-6 and IL-10 cytokine levels were measured in the supernatant by ELISA. Data shown as ratio of the median value of secreted cytokine following stimulation with both a specific TLR agonist and *C. difficile lgt* PGN over the sum value resulting from stimulation with each agonist alone. Values represent mean ± SEM (n>=3) analysed in duplicate.

4.2.3 Chronic stimulation with *C. difficile* PGN mediates tolerance to LPS

So far, our work has focused on the outcome of acute, rather than chronic, PGN stimulation. We became interested in the effect of chronic PGN stimulation on MDMs and particularly the immune outcome to sequential stimulation with LPS. For this study, primary monocytes were treated with 1µg/ml *lgt C. difficile* PGN for 24h, followed by culture in fresh media containing 10% autologous serum for 5d. At day 6, MDMs were stimulated with LPS for 24h prior to analysis of TNFα and IL-10 cytokines in the supernatant. Remarkably, chronic stimulation with PGN led to a significant reduction in the TNFα response to LPS (P=0.0013) (Figure 4.12). Likewise, we observed a statistically significant, yet less pronounced, decrease in induction of IL-10 (P=0.0315). It is worth noting that pre-treatment with PGN did not lead to spontaneous cytokine induction. This evidence suggests that PGN from *C. difficile* can induce differentiating monocytes to adopt a tolerogenic profile as mature APCs.

To gain more evidence of how chronic stimulation with PGN could alter the functional fate of MDMs, we treated monocytes with *C. difficile* PGN prior to their *in vitro* differentiation into MDMs. We then analysed the expression of various surface molecules in response to LPS. First, we looked at the expression of co-stimulatory proteins. CD54, a marker of macrophage activation (328), was included along with the markers studied earlier (see Figure 4.5). Importantly, we did not detect any significant difference in surface protein expression between untreated and PGN-pre-treated MDMs (Figure 4.13). LPS-treated MDMs expressed > 5 times more CD54 and CD86 on their surface. CD80 levels were also higher but only by ~ 2-fold. Remarkably, CD54 and CD86 up-regulation was significantly enhanced in cells that had been pre-treated with PGN by nearly 2-fold.

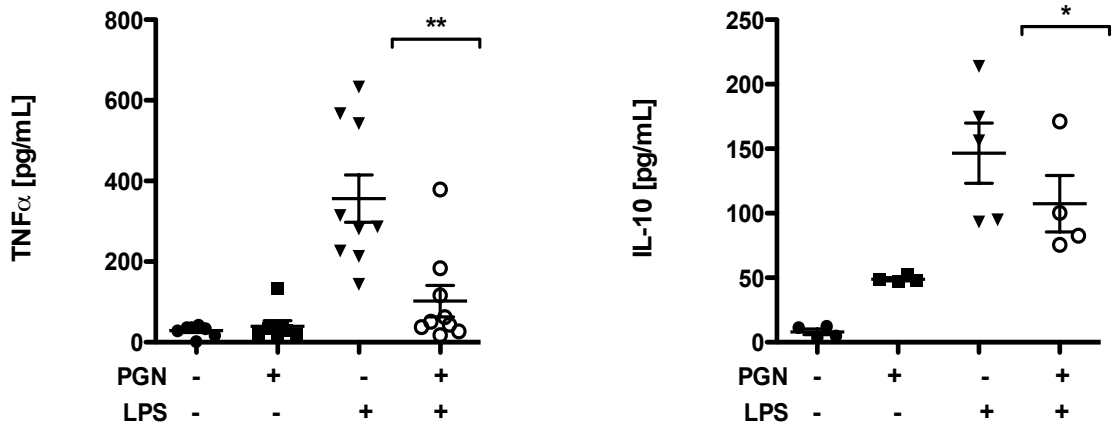

Figure 4.12. *C. difficile* PGN is an effector of trained immunity in human MDMs. $5x10^5$ CD14+ monocytes were left untreated or stimulated with 1µg/ml *C. difficile lgt* PGN for 24h in RPMI with 10% autologous serum. After 24h, cells were cultured in fresh media with 10% autologous serum for another 72h, prior to stimulation with 10ng/ml LPS. 24h post-stimulation, TNFα cytokine was measured in the supernatant by ELISA. Values represent means ± SEM from a minimum of three donors. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

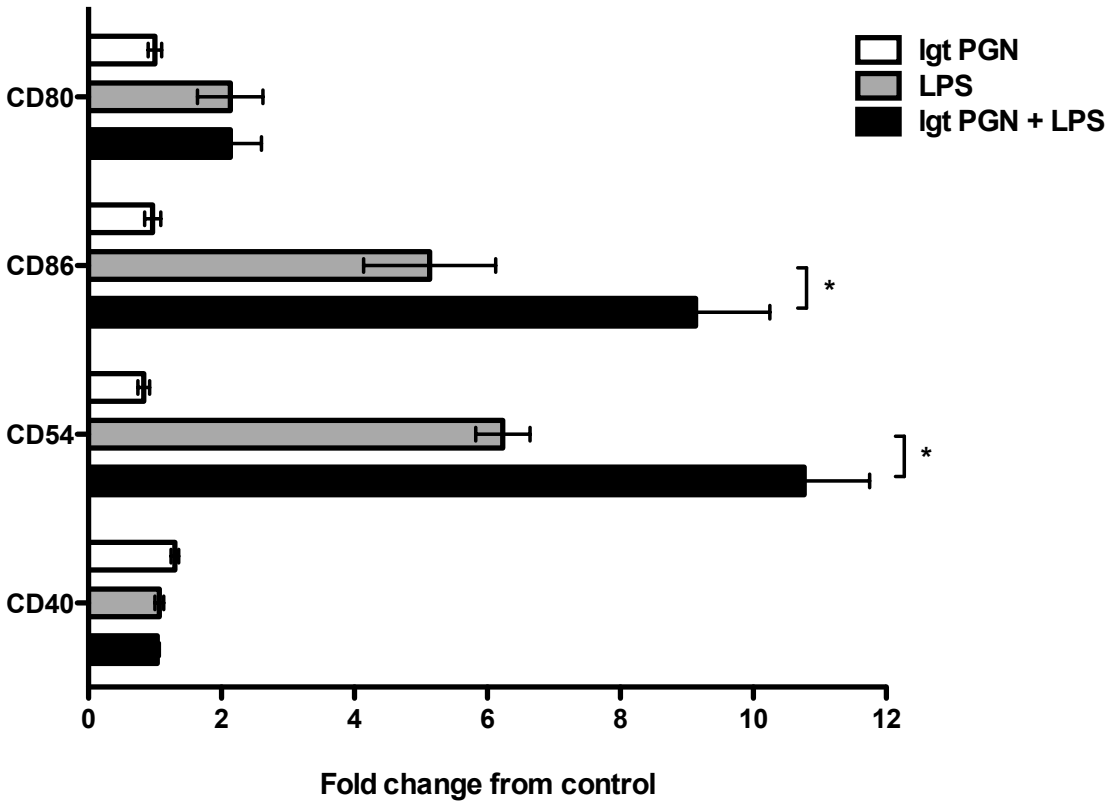

Figure 4.13. *C. difficile* PGN pretreatment affects the LPS-mediated co-stimulatory protein expression in human macrophages.

$2x10^5$ MDMs were left untreated or stimulated with 1µg/ml *C. difficile* PGN for 24h in media containing 10% autologous serum. After 24h, cells were cultured in fresh media with 10% autologous serum for another 72h, prior to stimulation with 10ng/ml LPS. 24h post-stimulation, expression of various co-stimulatory proteins was analysed by flow cytometry. Values represent means ± SEM from four donors. Statistical analysis by paired t-test (*$P<0.05$).

Subsequently, we analysed the effect of chronic *C. difficile* PGN stimulation on the expression of immune receptors in response to LPS. Our panel was expanded to include CD14, a co-receptor of TLR2 and TLR4 (21), and CD16, an Fc receptor involved in antibody-dependent cytotoxicity (329). CD14 is highly expressed in monocytes but not macrophages, while the opposite is true for CD16; allowing us to assess the extent of differentiation the cells have undergone. We also analysed the expression of the pan-macrophage marker CD11, a component of integrin receptors involved in various immune phenomena (330). Furthermore, the chemokine receptors CCR4, prominent in M2-type macrophages(331) and CCR7, expressed in M1-type macrophages were also studied. Macrophages may undergo classical M1 activation or alternative M2 activation into states that mirror the Th1–Th2 polarization of T cells (332). Again, we did not detect any significant difference in surface protein expression between untreated and PGN-pre-treated MDMs (Figure 4.14). LPS treatment induced up-regulation of TLR2 and CD14, although *C. difficile* PGN had no effect on TLR2 expression, similar to our findings in monocytes (see Figure 4.8). Pre-treatment with *C. difficile* PGN enhanced the up-regulation of CD14 from nearly 6-fold to 9-fold, despite the results not being statistically significant, possibly due to inter-donor variation. CCR4 expression was ~ 5x higher in untreated and ~ 9x higher in *C. difficile* PGN pre-treated cells after LPS stimulation, suggesting that PGN enhances the expression of the receptor. Yet, similar to CD14, the effect was not statistically significant. CCR7 was up-regulated by ~ 3-fold in both untreated and PGN-pre-treated cells. CD16 also became up-regulated in LPS-treated MDMs, yet we observed significant down-regulation of LPS-induced CD16 expression in PGN pre-treated cells. Overall, receptor up-regulation following treatment with LPS is consistent with the maturation on naïve immune cells to APCs. Pre-treatment with *C. difficile* PGN had a significant effect on proteins that showed high inducibility by LPS. CD16 was the only exception, as chronic stimulation

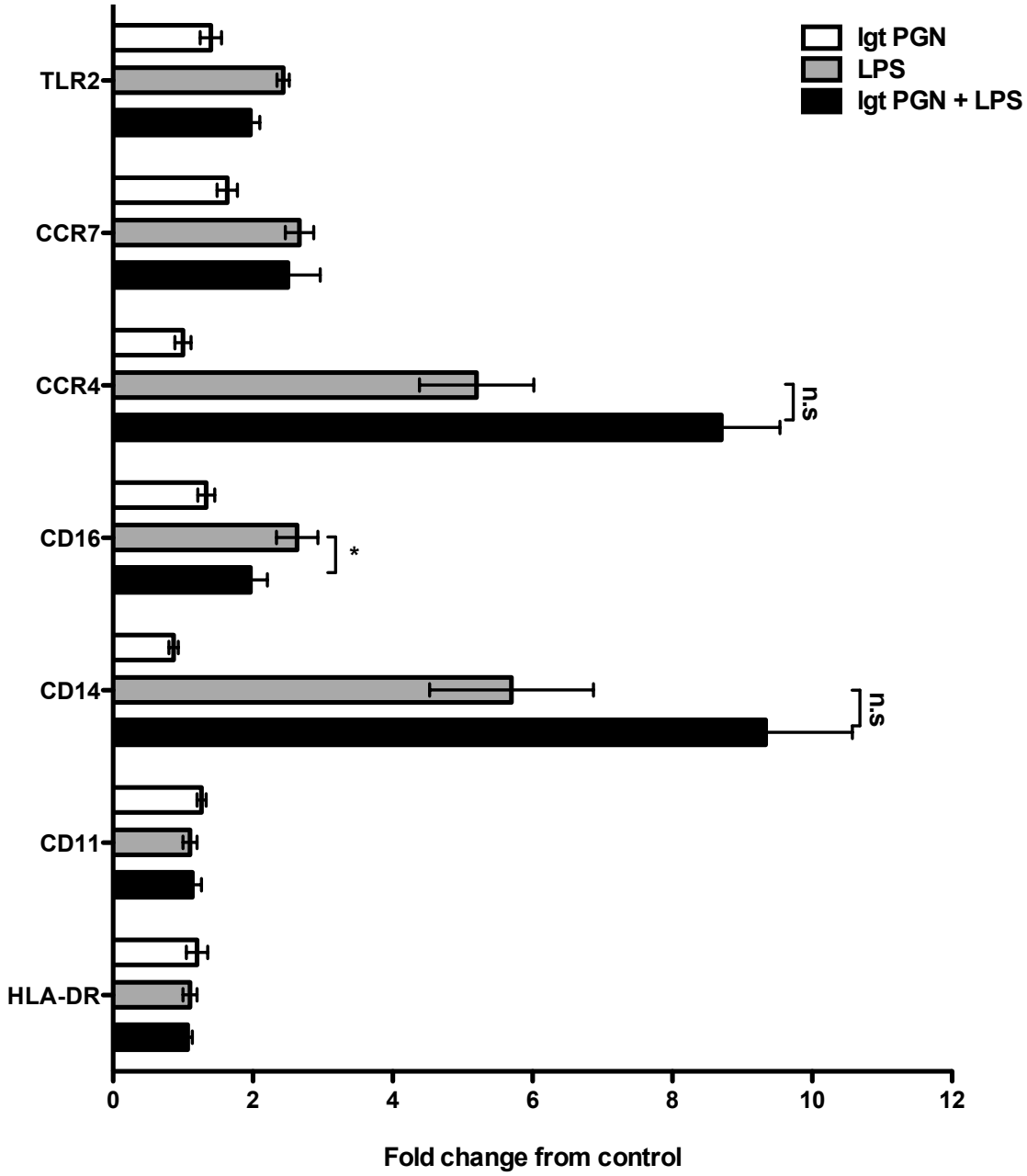

Figure 4.14. *C. difficile* PGN pretreatment affects the LPS-mediated receptor up-regulation in human macrophages.

$2x10^5$ MDMs were left untreated or stimulated with 1µg/ml *C. difficile* PGN for 24h in media containing 10% autologous serum. After 24h, cells were cultured in fresh media with 10% autologous serum for another 72h, prior to stimulation with 10ng/ml LPS. 24h post-stimulation, expression of various markers was analysed by flow cytometry. Values represent means ± SEM from four donors. Statistical analysis by paired t-test (*P<0.05, n.s P>0.15).

with *C. difficile* PGN led to a decrease in the LPS-mediated induction of the receptor.

4.5 Discussion

In this chapter, the immunogenicity of *C. difficile* PGN with regard to the cytokine response and phenotypic changes elicited in monocytes and MDMs was examined. Compared to the PGN of *S. aureus*, *C. difficile* PGN induced higher levels of IL-8 (Figure 4.1), indicating a propensity for inflammatory induction. Whether the difference in IL-8 is the result of different PGN isolation methods or lipoprotein content remains unknown. On the other hand, only low levels of TNFα or IL-10 were detected (Figure 4.1), suggesting that *C. difficile* PGN does not activate the TNFα/IL-10 signalling axis. This may further contribute to the inflammatory response to PGN, as the TNFα/IL-10 signalling axis is known to regulate mucosal immunity (333) and limit intestinal inflammation. TNFα, an early-response cytokine, was only detected at significant levels after 24h. This discrepancy may be explained by the requirement for internalisation and lysozyme digestion of the PGN prior to sensing by the NODs (286). IL-1β and IL-10 secretion in *C. difficile* PGN-treated cells was not significantly different from that of resting cells. Similarly, treatment of PBMCs with low dose DAP or MDP does not induce IL-1β or IL-10 in PBMCs (334),(307). Reports have shown that treatment of monocytes with *S. aureus* PGN results in high levels of IL-1β transcription, but not in secretion of the mature cytokine (324). Accordingly, we detected minimal levels of IL-1β from *C. difficile* PGN-treated cells (Figure 4.1). The low levels of secreted IL-1β reaffirm the lack of inflammasome activation reported in previous experiments (see Figure 3.13 in Chapter 3). Interestingly, the levels of secreted IL-8, IL-1β and TNFα were similar to those reported for *Bacillus anthracis* PGN (318). The effect of *S. aureus*

PGN on cytokine production by PBMCs was similar to that reported elsewhere (35),(335).

B. anthracis PGN is a potent stimulator of monocytes, neutrophils and B lymphocytes (286). Likewise, all three cell types were able to mount a response to *C. difficile* PGN in our experiments. Previous reports identified monocytes and neutrophils as the major sources of pro-inflammatory signalling in response to bacterial cell wall components (172). In our study, monocytes were the major contributors of IL-8 and TNFα amongst PBMCs (Figure 4.2 and 4.3), which is not surprising given that their TLR2 expression is many-fold higher compared to other blood cells (336),(337). Neutrophils were the second biggest contributors towards the cytokine response to PGN. It is worth noting that while NOD2 is expressed in both monocytes and neutrophils, NOD1 is only present in monocytes (69), which may lead to differential outcomes in response to PGN. Overall, TNFα levels were higher in comparison to other cytokines, reaffirming the role of TNFα as the major cytokine in the monocytic response to *C. difficile* PGN (318). As expected, the pro-inflammatory immune response was greatly affected by the lack of lipoproteins and, therefore, TLR2 signalling (Figure 4.2 and 4.3) (see Chapter 3). Studies on the PGN of other bacteria have reported a similar reduction in the pro-inflammatory immune response in the absence of lipoproteins (35),(320). On the other hand, induction of the anti-inflammatory cytokine IL-10 was not significantly different between WT and *lgt* PGN (Figure 4.4), contradictory to the central role of TLR2 in IL-10 expression (338). Yet, along with TLR2, PGN also activates NOD2 (62) which is also a key mediator of IL-10 expression (53).

Next, we looked at changes in the expression of both co-stimulatory molecules and receptors in response to *C. difficile* PGN. We found that up-regulation of differentiation markers in monocytes was lipoprotein-mediated and only affected the expression of

CD86 (Figure 4.5) and TLR2 (Figure 4.6). Lipoprotein-mediated up-regulation of TLR2 in monocytes has been reported previously (284). Although others have reported the PGN-mediated up-regulation of CD40 and CD80 expression in THP-1 cells (339), we did not observe a similar effect in monocytes. The expression of other markers traditionally associated with monocyte maturation was unaffected, suggesting that treatment with *C. difficile* PGN does not induce monocyte differentiation. Likewise, PGN from *S. aureus* failed to induce monocyte differentiation in a previous study (340).

We also looked at whether *C. difficile* PGN could modulate the expression of co-stimulatory molecules and receptors in response to LPS. Studies on MDP have shown it can enhance LPS-mediated up regulation of CD80, CD86 and CD40 in DCs (60),(69). However, we found no evidence to suggest modulation of molecule expression in the presence of *C. difficile* PGN (Figure 4.7 and 4.8). One explanation may be that the proteins analysed are predominantly expressed in mature APCs, such as macrophages and DCs (11), rather than monocytes. As such, the investigation could be repeated using a more appropriate set of markers. Additionally, since LPS is a strong inducer of monocyte maturation, any subtle effect may have gone undetected. As discussed earlier, mature APCs, such as macrophages, up-regulate PRR expression (341),(301) and may provide a better model for the study of PGN-mediated induction in receptor expression.

Since the study of cell surface factors did not provide any evidence of modulation of LPS signaling by *C. difficile* PGN, we examined the effect of the latter on the outcome of TLR4-mediated cytokine induction. Co-stimulation with PGN potentiated the production of TNFα in response to LPS (Figure 4.9) as well as other TLR ligands (Figure 4.10). Overall, the most significant contribution was towards TLR4 signalling, where we found evidence of synergistic activity between *C. difficile* PGN- and LPS-

mediated signaling (Figure 4.11). Accordingly, studies using MDP have found NOD2 signalling to potentiate TLR2, TLR3 and TLR4-mediated TNFα induction but not TLR5 (342), although a follow up study suggested the outcome was variable (327). Similar results were obtained for the induction of IL-6, with the exception of TLR3 where no modulation was detected (Figure 4.10). TLR3 and NOD ligands are weak inducers of IL-6 in monocytes (343) and any weak effect may have gone undetected. Finally, while PGN did not enhance IL-10 secretion in response to LPS (Figure 4.9), which could be explained by saturation of IL-10 induction by LPS alone, the IL-10 response to all other TLR ligands was enhanced (Figure 4.10). Notably, IL-10 enhancement was more prominent in the presence of the TLR5 ligand flagellin (Figure 4.11), similar to a previous study with MDP (327). There are contradictory reports regarding the IL-10 response to flagellin (344) as it has been reported elsewhere that flagellin does not induce IL-10 production in murine cells (345). Isolated and purified flagellin was used in those studies; therefore it is possible that the commercially available preparation used may contain contaminants.

To assess whether the detected modulation of cytokine induction was due to a synergistic effect of *C. difficile* PGN with TLR ligands, the cytokine production after stimulation with both ligands was compared to the outcome of stimulation with each ligand alone. Synergy between NOD2 and TLR2 (307),(342),(320) or TLR4 (346),(69),(61) signalling has been reported in multiple studies. While *C. difficile* PGN was able to modulate TLR2-mediated cytokine induction, we did not detect evidence of synergy. The use of another TLR2 agonist with no NOD-stimulating activity, such as Pam3CSK4 may have been more appropriate. Finally, the study could be extended to other cytokines and chemokines, such as IL-8. For example MDP- and DAP-containing desmuramylpeptides in combination with chemically synthesized TLR agonists can synergistically induce IL-8 production in a NOD2- and NOD1-dependent manner,

respectively (307). Further research into the complex crosstalk amongst these receptors is needed to understand their role in orchestrating the maintenance of a balanced pro-/anti-inflammatory axis.

The long-term effects of PGN stimulation on innate immune cells were also investigated. Presently, two opposing narratives exist on the outcome of chronic NOD stimulation. On the one hand, studies have shown that long-term NOD2 stimulation in macrophages leads in tolerance to LPS re-stimulation and a diminished cytokine response (81). On the other, NOD ligands are reported as effectors of trained immunity which offers a protective effect to macrophages by enhancing their cytokine response to sequential LPS stimulation (84). We found evidence of cross-tolerance to LPS upon chronic stimulation with *C. difficile* PGN (Figure 4.12). Hedl *et al.* first reported that prolonged exposure of primary MDMs to MDP inhibited both the NOD2- and TLR4-mediated pro-inflammatory response (81). Intriguingly, the same study reported that intestinal macrophages exhibit tolerance to MDP and the TLR4 agonist lipidA. The same group later demonstrated that NOD2-mediated induction of IL-10 led to the suppression of MAPKs and transcription factors controlling the expression of pro-inflammatory cytokines (347). It would be interesting to study the effect of *C. difficile* PGN on MAPK signalling, as, others have shown that the pathway plays a key role on the inflammatory response to *B. anthracis* PGN (318). We also examined the production of IL-10, where Hedl et al. have reported that chronic NOD2 stimulation down-regulated IL-10 secretion upon TLR4 activation (82); mirroring our own findings (Figure 4.12). Finally, although we did not find evidence of “trained immunity” in PGN-treated MDMs, it is worth noting that the outcome of a previous study (322) was highly dependent on the concentration of agonist used and would require use of PGN at significantly higher concentration.

In an attempt to characterise the functional reprogramming of MDMs following chronic stimulation with *C. difficile* PGN, we analysed the expression of various surface molecules by flow-cytometry. Treatment of monocytes with PGN did not affect the expression of co-stimulatory factors or receptors in the differentiated MDMs (Figure 4.13 and 4.14) and we are not aware of any studies that have shown differential marker up regulation in macrophages in response to chronic stimulation with PGN. As expected, we observed up-regulation of a range of factors involved in immune surveillance following LPS stimulation. The most predominant increase was in the levels of the co-stimulatory molecules CD54, CD80 and CD86 (Figure 4.13), and the receptors CD14 and CCR4 (Figure 4.14). Previous studies have reported on the up-regulation of CD54 (348), CD80 and CD86 (349), as well as CD14 (350) and CCR4 (351) in response to LPS.

In cells pre-treated with *C. difficile* PGN, we observed significant enhancement of both CD54 and CD86 expression (Figure 4.13). A previous study also reported enhanced expression of CD54 and CD86 after co-stimulation with LPS and the NOD1 agonist DAP (69). While little is currently known on the function of CD54 in macrophages (352), it plays a role in the macrophage-mediated Th2 polarisation of T cells (353). Likewise, CD86 mediates T cell activation by macrophages to induce a Th2 response (354). The Th2-type response induces the production of anti-inflammatory cytokines, such as IL-10, and is considered tolerogenic. There was also an increase in the levels of CD14 and CCR4 expression after LPS in PGN-pre-treated cells compared to untreated (Figure 4.14). CD14, although considered a monocytic marker, is also expressed in macrophages (355). Monocytes expressing both CD14 and CD16 are considered pro-inflammatory, as opposed to those that express CD14 alone (356). In our study, while the expression of CD14 was enhanced, CD16 expression was lower in cells pre-treated with *C. difficile* PGN. Following the paradigm of monocytes, this may concur with the

adoption of a more tolerogenic profile. Finally, CCR4 is recognised as a key receptor in Th2-associated processes (331). Furthermore, it has been involved in the activation of PI3K signalling, one of the key pathways driving IL-10 transcription (357).

Therefore, a pattern of more tolerogenic, Th2-like response to LPS in MDMs pre-exposed to *C. difficile* PGN is emerging. These findings complement our work showing a reduced pro-inflammatory cytokine response in these MDMs, which more closely resembles M2-type macrophages (Figure 4.12). In future work, M2-type polarisation would have to be assessed by a separate set of markers such as CX3CR1, CD163 and CD206 (358). Furthermore, since M2-type polarisation does not lead to down-regulation of M1-type factors (359), further analysis of markers in this category is required. It may be worth noting, however, that a recent evaluation of the features of macrophage activation in response to bacterial infection concluded that the use of the polarisation concept has been mostly unsuccessful (360). In the greater scheme, studies of this nature are important for the development of vaccine adjuvants and recently, peptide mimics of PGN have been identified as vaccine candidates (361). Studying the signalling pathways activated by *C. difficile* PGN and how they modulate control of cytokine expression at the promoter level could help unravel the mechanism of PGN-mediated torerance.

Chapter 5

Immunomodulation by *Campylobacter jejuni* flagellum

5.1 Introduction

Published work from our group recently identified *C. jejuni* strain 11168H as a potent inducer of IL-10 and described the role of flagellum in the IL-10 response to this pathogen (218). Enhancement of IL-10 was flagellum-specific, as the use of a secretion positive (*ΔflaA*) and secretion negative (*ΔrpoN*) flagella mutant strains revealed that proteins secreted from *C. jejuni* flagella played no role in IL-10 induction. Furthermore, pseudaminic acid (Pse) residues attached to the flagellum appeared to modulate IL-10 induction in human DCs. According to the study, Pse residues are recognised by the Siglec10 receptor, which was shown to mediate flagellum- specific enhancement of the IL-10 response to *C. jejuni*. IL-10 response to the pathogen was MAPK-dependent and the increase in IL-10 expression was driven by hyper-activation of the MAPK signalling pathway, specifically of ERK and p38 kinase activation. Focusing on the IL-10 response to *C. jejuni*, we wanted to study the signalling events that mediate flagellum-dependent enhancement of IL-10 production.

5.1.2 Regulation of IL-10 expression

In vivo expression of IL-10 following infection has been reported in macrophages, DCs and neutrophils (338). Both macrophages and DCs can express IL-10 *in vitro* following PRR activation by microbes, although the response is much higher in macrophages. TLR2 ligands are considered as specialised IL-10 inducers (362), although significant amounts of IL-10 are produced following TLR4 and TLR9 activation (363). On the other hand, TLR5 signalling is a negative regulator of IL-10 expression (345). TLR-independent induction of IL-10 mediated by NOD2 has also been reported (53). Finally, C-type lectin or Fc receptor (FcR) ligands can also mediate TLR-independent IL-10 induction (364).

Following TLR ligation, Toll/IL-1 receptor (TIR)-domain-containing adaptors, such as myeloid differentiation primary response protein 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFNβ (TRIF), mediate signalling cellular activation. MyD88 and TRIF dual activation leads to optimal IL-10 induction by LPS. MyD88 activation leads to phosphorylation of MAPKs and NF-κB (32). The MAPK cascade is composed of three major groups of kinases: extracellular signal-regulated kinases (ERKs) comprising ERK1 and ERK2; JuN N-terminal kinases (JNKs) comprising JNK1 and JNK2; and p38 (272). ERK activation is key to the modulation of IL-10 expression (365) and differences in IL-10 production between macrophages and DCs correlate with the strength of ERK activation in each cell type (366). Following TLR stimulation, ERK is most highly activated in macrophages, with lower activation in DCs. IL-10 expression is also dependent on p38 signalling in macrophages, primary DCs and human peripheral blood monocytes (367). ERK and p38 signalling consolidate in the activation of the MSK1 and MSK2 kinases, which in turn activate transcription factors that bind the IL-10 promoter. The transcription factors cAMP-responsive-element-binding protein (CREB) and activating transcription factor 1 (ATF1) have been shown to be activated by MSKs in LPS-stimulated mouse macrophages (278). In addition, IL-10 production is regulated by the phosphoinositide 3-kinase (PI3K)–AKT pathway, which antagonises the activation of glycogen synthase kinase 3 (GSK3), a negative regulator of IL-10 (Figure 5.1).

Downstream of these cascades, IL10 expression depends on multiple transcription factors, including specific protein 1 (SP1) and SP3, IFN-regulatory factor 1 (IRF1) and STAT3 (338). Although no evidence of direct binding of the various STATs to the IL10 locus currently exists, STAT proteins are increasingly being implicated in regulating the induction of IL-10 expression in both primary macrophages and T cells. Along with

these distal regulatory elements, transcription factors that bind regions of the locus outside the IL-10 promoter region and become activated via ERK signalling have also been discovered (362).

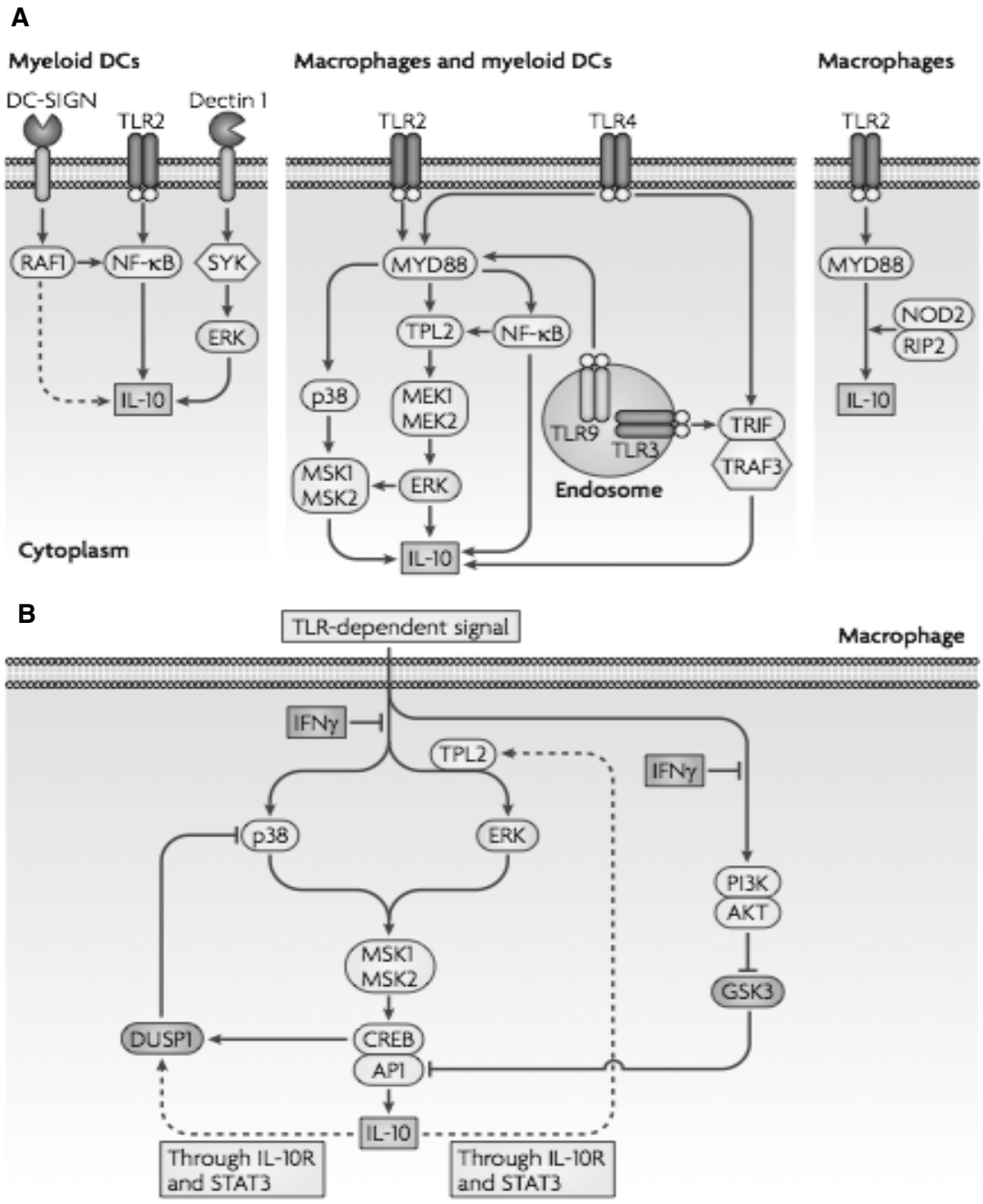

Figure 5.1. Induction of IL-10 expression in innate immune cells.

(A) TLR-dependent and TLR-independent IL-10 induction in myeloid DCs and macrophages. (B) Positive and negative feedback loops in IL-10 induction in macrophages

5.2 Results

5.2.1 *C. jejuni* flagellum-mediated enhancement of IL-10 production

Our group was the first to identify pathogenic strains of *C. jejuni* as potent inducers of the anti-inflammatory cytokine IL-10 and the role of flagella in that response (218). The work was carried out primarily in mouse bone marrow-derived DCs (BMDCs), and we have since wanted to examine whether the findings are also true in human cells. Human monocyte-derived DCs (mDCs) were co-cultured with two pathogenic strains of *C. jejuni* (11168H and 81-176) to study the cytokine response to the pathogen. Co-culture with *C. jejuni* 11168H at two different MOIs (10 and 100) led to induction of IL-10 with a median ~ 700 and 2100pg/ml respectively after 24h (Figure 5.2). A similar dose-dependent increase in TNFα was observed with cytokine levels at a median ~ 30 and 58 ng/ml. *C. jejuni* 81-176 appeared to induce less IL-10 and TNFα at MOI 100, with a median ~1300pg/ml and 45 ng/ml respectively, however the dose-dependent increase in cytokine levels was comparable between the two strains. We also examined the timing of cytokine induction by co-culturing mDCs with *C. jejuni* for 8 and 24h. IL-10 levels of only ~ 280pg/ml were detected at 8h, but were much higher with a median ~ 1900pg/ml by 24h (Figure 5.3). High TNFα levels were detected both after 8h and 24h of co-culture with *C. jejuni*, with a median ~ 17 and 54 ng/ml respectively. A similar time-dependent effect on cytokine induction was observed in cells co-cultured with *C. jejuni* 81-176. Based on these findings, a model for *C. jejuni* co-culture with human APCs using bacteria at MOI 100 for 24h was established.

Subsequently, we set out to investigate the role of flagellum in *C. jejuni*-mediated cytokine induction. It has been reported that some ε-proteobacteria, including *C. jejuni*, have evolved to evade TLR5 recognition (217). To confirm that the flagellum of *C. jejuni* strain 11168H does not activate TLR5, WT and TLR5-transfected HEK293 cells were

co-cultured with WT and *ΔFlaA C. jejuni* strain 11168H for 24h. *S. aureus*, which also belongs to the proteobacteria taxa and does not activate TLR5, was used as a control. WT and flagellum-negative *FliC Salmonella* typhimurium, as well as flagellin derived from the pathogen, were used to confirm flagellum-specific TLR5 activation. Induction of IL-8 was minimal in WT HEK293 cells across all treatments (Figure 5.4). Activation was only detected in *Salmonella* typhimurium-infected and flagellin-treated TLR5 HEK293 cells, while infection with *C. jejuni* or *ΔFliC Salmonella* typhimurium did not induce IL-8 expression, confirming that *C. jejuni* flagellum is TLR5 insensitive.

Our lab had previously demonstrated that the IL-10 response to *C. jejuni* is modulated in the presence of the flagella (218). The critical role of flagella in the induction of IL-10 in human APCs was confirmed by co-culturing mDCs as well as M1- and M2-type macrophages with WT or *ΔFlaA C. jejuni* 1168H. While the TNFα response was unaffected by the lack of flagella, IL-10 levels were significantly lower in cells co-cultured with the *ΔFlaA* mutant; both DCs (Figure 5.5; $P=0.037$) and M1-type ($P=0.039$) or M2-type ($P<0.001$) macrophages (Figure 5.6). Notably, the difference in IL-10 induction was more pronounced in M2-type macrophages, which are considered anti-inflammatory.

The lack of a model cell line for human DCs led as to utilise pre-monocytic THP-1 cells which were PMA-treated to achieve macrophage-like differentiation into dTHP-1 cells, as discussed in Chapter 3. We also considered macrophages to be a more appropriate model as they produce significantly higher levels of the cytokine compared to DCs. The contribution of flagella in the cytokine response of these cells to *C. jejuni* was analysed to assess whether the cell line would offer an appropriate model for our investigation. IL-10 gene expression was analysed from 1h following co-culture with *C. jejuni* 11168H and up to 24h (Figure 5.7). IL-10 and TNFα cytokine levels were also measured at 8h

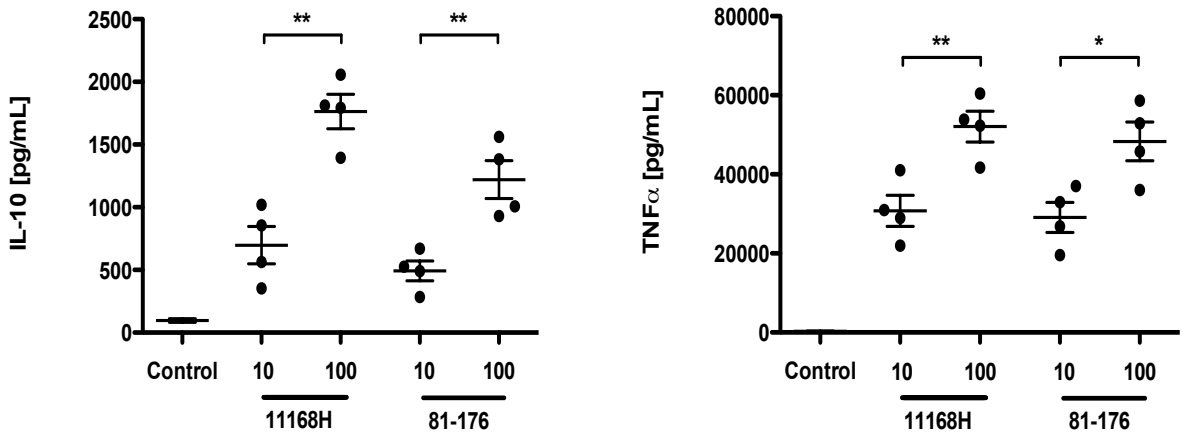

Figure 5.2. Dose-dependent effect of *C. jejuni* on monocyte-derived dendritic cell (mDC) IL-10 and TNFα production.

$5x10^5$ human monocyte-derived dendritic cells (mDCs) were co-cultured with wild-type (WT) *C. jejuni* strains 11168H or 81-176 at MOI 10 or 100 for 24h. IL-10 and TNFα cytokines were analysed in the supernatants by ELISA. Values represent means ± SEM from four donors measured in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<001).

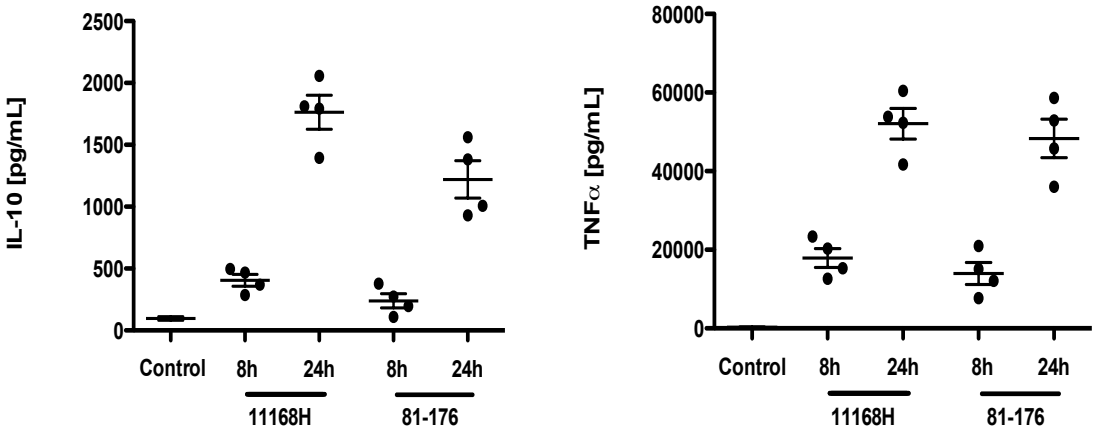

Figure 5.3. Time-dependent effect of *C. jejuni* on mDC IL-10 and TNFα production. $5x10^5$ human mDCs were co-cultured with *C. jejuni* strain 11168H or 81-176 at MOI 100 for 8 and 24h. IL-10 and TNFα cytokines were analysed in the supernatants by ELISA. Values represent means ± SEM from four donors measured in duplicate.

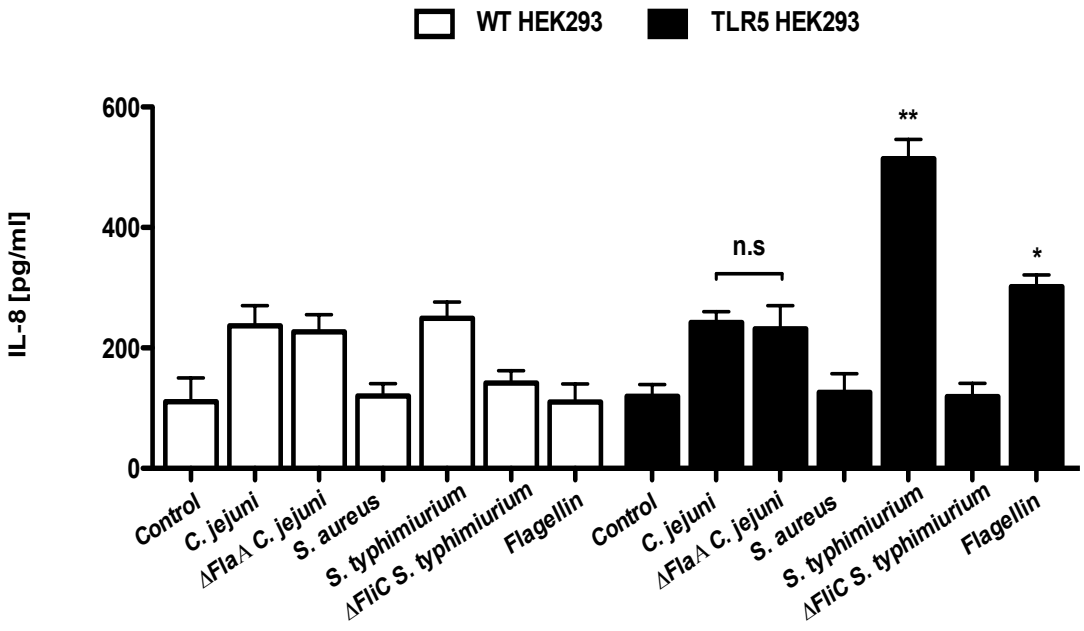

Figure 5.4. C. jejuni flagellum is not recognised by TLR5.

1x10^6 WT or TLR5-transfected HEK cells were co-cultured with clinical isolates of various enteropathogens; WT or *ΔflaA C. jejuni* strain 11168H (MOI 100), *S. aureus (*MOI 5) and WT or *ΔFliC Salmonella t*yphimurium (MOI 1). 5µg/ml flagellin from *Salmonella* typhimurium was used as a positive control of TLR5 activation. After 6h, IL-8 was analysed in the supernatants by ELISA. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

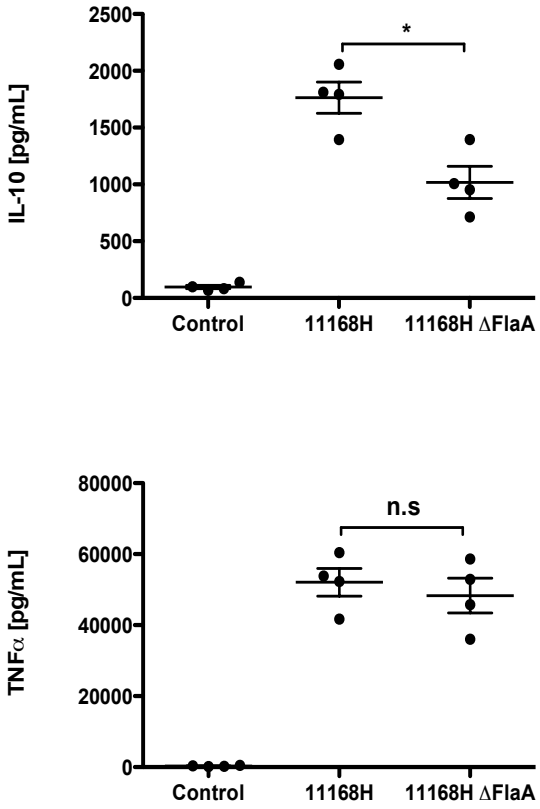

Figure 5.5. *C. jejuni* flagellum is required for optimal IL-10 induction in human monocyte-derived DCs.

5x105 human mDCs were co-cultured with WT or ΔFlaA *C. jejuni* strain 11168H MOI 100. After 16h, IL-10 and TNFα cytokines were analysed in the supernatants by ELISA. Values represent means ± SEM from four donors measured in duplicate. Statistical analysis by paired t-test (*P=0.0127).

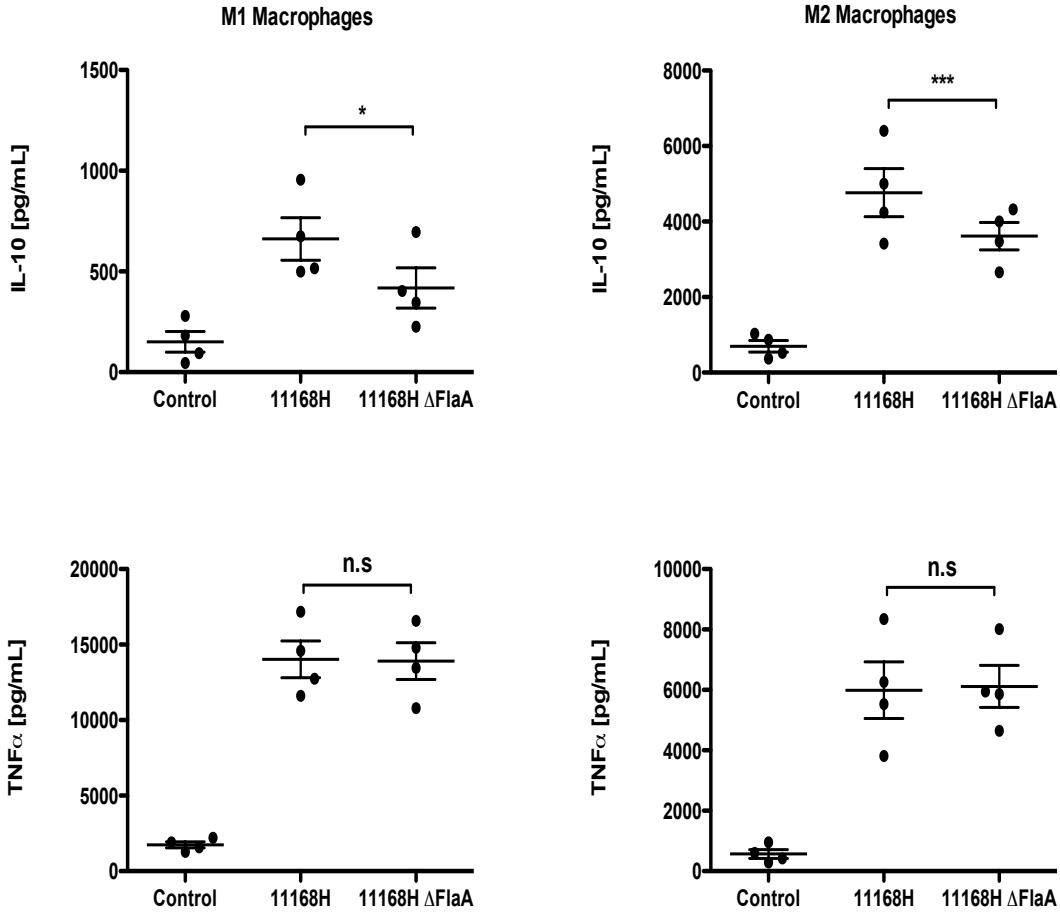

Figure 5.6. *C. jejuni* flagellum is required for optimal IL-10 induction in human MDMs.

$5x10^5$ human monocyte-derived M1- and M2-type macrophages were co-cultured with WT or *ΔflaA C. jejuni* strain 11168H MOI 100. After 16h, IL-10 and TNFα cytokines were analysed in the supernatants by ELISA. Values represent means ± SEM from four healthy donors analysed in duplicate. Statistical analysis by paired t-test (*P=0.045, ***P=0.0001).

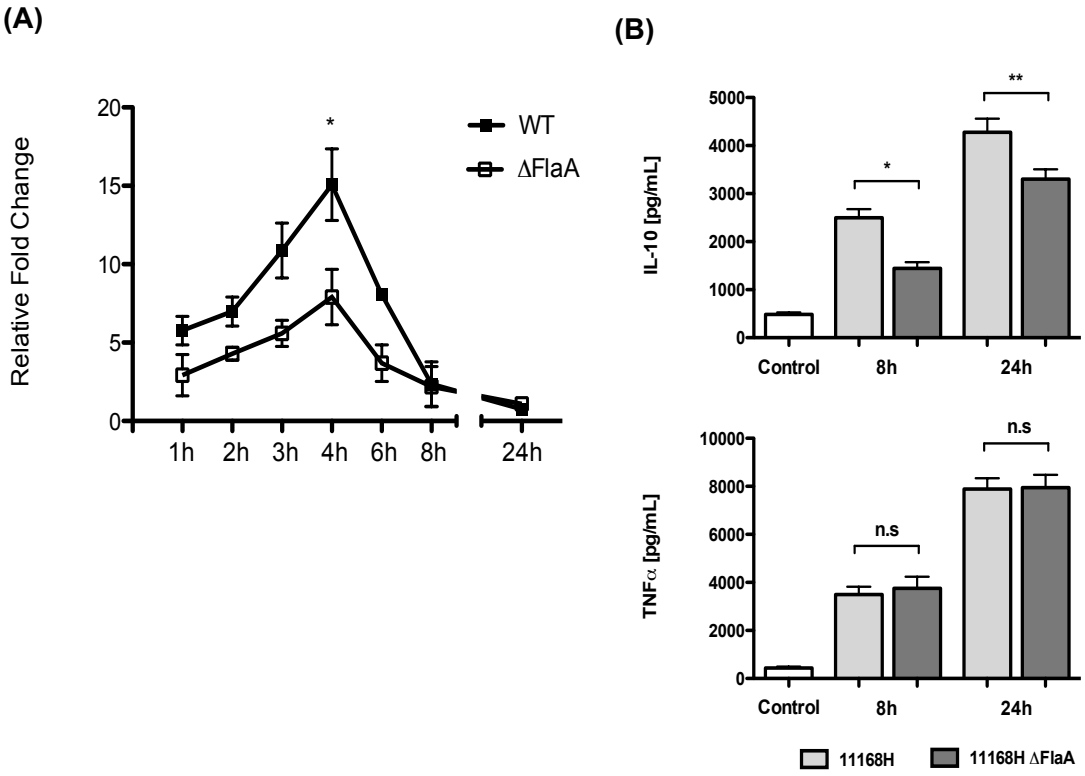

Figure 5.7. *C. jejuni* flagellum is required for optimal IL-10 induction in dTHP-1 cells.

5x10^5 dTHP-1s were co-cultured with WT or *ΔFlaA C. jejuni* strain 11168H MOI 100 for 8 and 24h. (A) At the indicated times, IL-10 gene transcription was analysed by quantitative real-time PCR. Values are expressed as mean ± SEM from three independent experiments (*P=0.05). (B) IL-10 and TNFα cytokines were analysed in the supernatant by ELISA. Values are means ± SEM from three independent experiments analysed in duplicate. Statistical analysis by paired t-test (*P=0.0338, **P=0.0056).

and 24h post-infection. Differential induction of IL-10 transcription in dTHP-1 cells co-cultured with WT and *ΔFlaA C. jejuni* was observed consistently from 1h and up to 8h following infection (Figure 5.7A) with the difference becoming statistically significant at 4h (P=0.047). Correspondingly, a statistically significant difference in IL-10 protein levels was observed at both 8 (P=0.034) and 24h (P=0.006) (Figure 5.7B). Similar to primary APCs, no difference was observed in TNFα protein levels (Figure 5.7C).

To study the early-stage interactions of *C. jejuni* with macrophages, the rate of bacterial phagocytosis following adherence was also examined by quenching extracellular fluorescence from FITC-labelled bacteria, thus detecting only cells where intracellular bacteria were present. As Figure 5.8 shows, bacteria adhered to ~ 20% of cells after 15mins of co-culture but only became internalised by ~ 3% of those cells. After 30mins, however, intracellular bacteria were detected in over 19% of cells from a total of ~ 30% positive for *C. jejuni*. And after 60mins of co-culture, that number had risen to ~ 33% of cells from a total of 40% of cells with bacteria present. Therefore, *C. jejuni* is rapidly internalised by dTHP-1 cells and comes early in contact with the cellular immune apparatus. After 90mins the numbers of total and intracellular bacteria did not change significantly. This information is important for the study of *C. jejuni* survival within macrophages.

We also argued that lack of flagella could affect the motility and hence the interaction of the *ΔFlaA C. jejuni* mutant with macrophages. In addition to comparing WT and *ΔFlaA C. jejuni*, immotile heat-inactivated *C. jejuni* were compared to the live, motile bacteria. Live or heat-inactivated, WT or *ΔFlaA C. jejuni* was FITC-labelled prior to co-culture with dTHP-1s. The experiment was performed either at 4°C to prevent bacterial internalisation or at room temperature where phagocytosis could take place. No significant difference in the adherence of WT and *ΔFlaA C. jejuni*, live or heat-

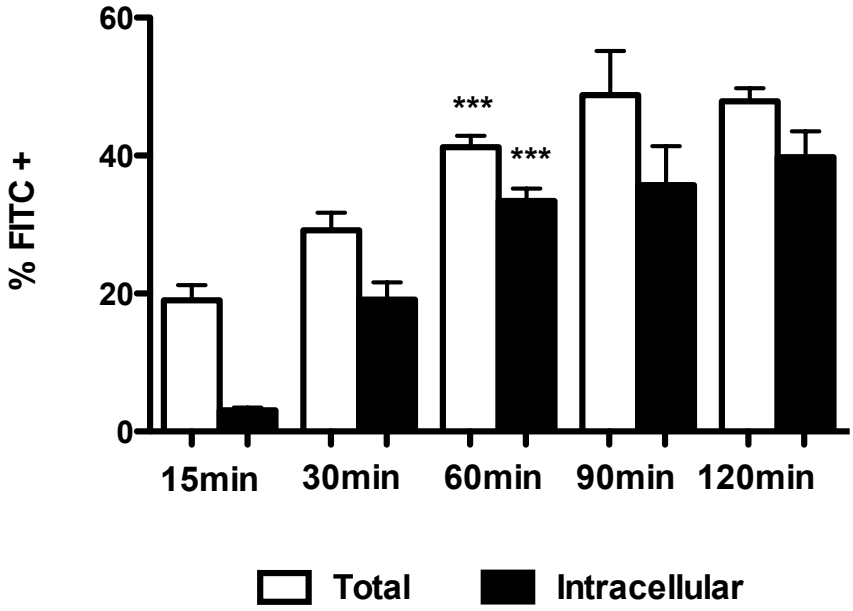

Figure 5.8. Time-dependent phagocytosis of *C. jejuni* by dTHP-1 cells.

$5x10^5$ dTHP-1 cells were co-cultured with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 for the indicated times. After the presence of bacteria was analysed by flow cytometry, trypan blue was used to assess the presence of intracellular bacteria. Values are means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis by paired t-test (***P< 0.0001).

inactivated was detected (Figure 5.9). Heat-inactivated bacteria appeared to adhere slightly better than live, with ~ 61 compared to 58% FITC-positive cells, perhaps due to the lack of a capsule which may reveal structures recognised by dTHP-1 cells. Likewise, we did not observe a significant difference in the internalisation of WT and *ΔFlaA C. jejuni* (Figure 5.10). Additionally, the survival assay performed showed no difference in *C. jejuni* survival between the WT and *ΔFlaA* mutant (Figure 5.11), indicating that flagellum and motility do not affect the intracellular life of the microorganism.

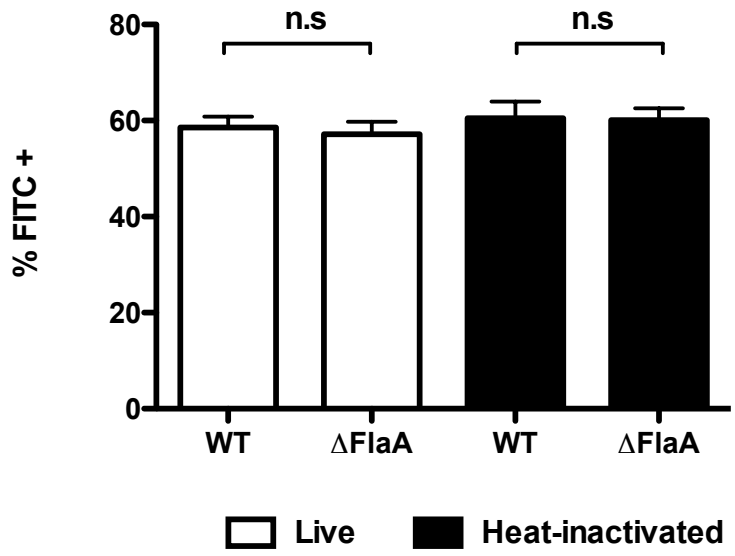

Figure 5.9. Flagella do not affect *C. jejuni* adherence to dTHP-1 cells. 5x10^5 dTHP-1s were co-cultured with FITC-labelled live or heat-inactivated WT or *ΔFlaA C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, bacterial adherence was analysed by flow cytometry. Values are means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis by paired t-test.

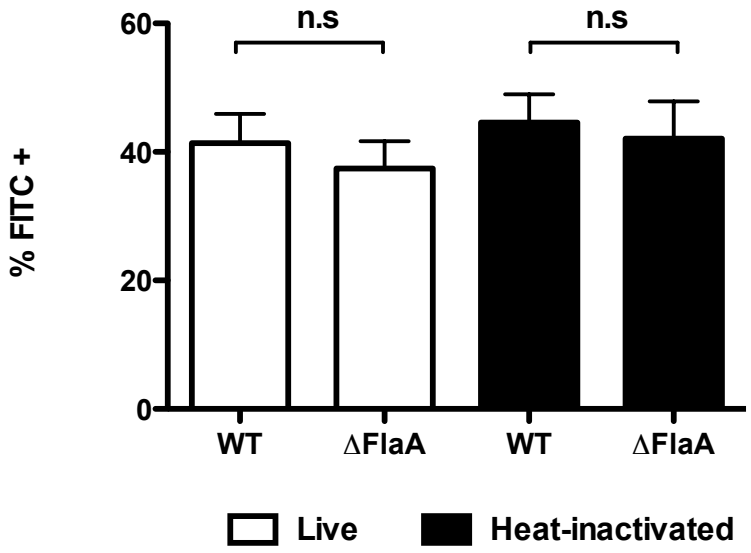

Figure 5.10. Flagella do not affect *C. jejuni* phagocytosis by dTHP-1 cells.
$5x10^5$ dTHP-1 cells were co-cultured with FITC-labelled live or heat-inactivated WT or *ΔFlaA C. jejuni* strain 11168H MOI 100 for 1h. Intracellular bacterial presence was assessed using trypan blue dye and flow cytometry. Values are means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis by paired t-test.

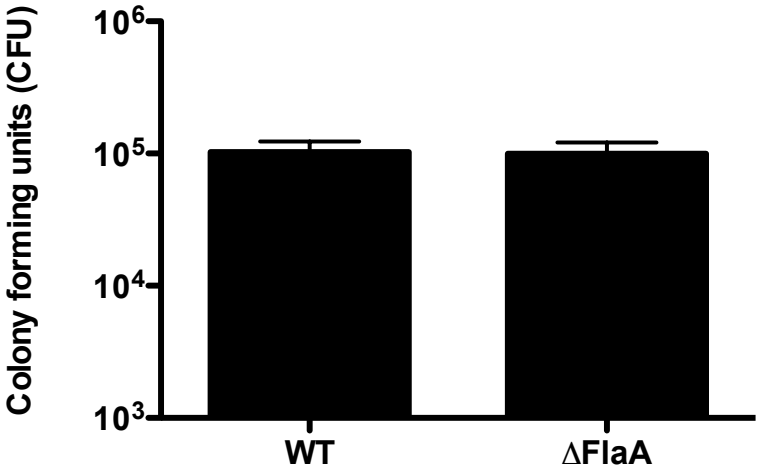

Figure 5.11. Flagella do not affect *C. jejuni* survival in dTHP-1 cells.

$5x10^5$ dTHP-1s were co-cultured with WT or *ΔFlaA C. jejuni* strain 11168H MOI 100 for 2h, followed by gentamycin treatment (100μg/ml; to kill extracellular bacteria) for an additional 2h. 4h post-infection, cells were lysed and intracellular bacteria plated and colonies counted after 48h. Values are mean ± SEM from at least three independent experiments performed in duplicate.

5.2.2 Differential signalling in the presence of *C. jejuni* flagella

Once the role of the flagella in the *C. jejuni*-mediated IL-10 response had been established; we set out to characterise the signalling mechanisms underlying the differential cytokine induction. Previous work based in BMDCs established that flagellum-mediated signalling is NF-κB independent (218). We confirmed these findings in a model of human macrophages by transducing dTHP-1 cells with a reporter construct for NF-κB activation; no difference was observed between WT and *ΔFlaA C. jejuni*-induced signalling (Figure 5.12). Instead, differential induction of IL-10 in the presence of flagella was attributed to hyper-activation of MAPK signalling; more specifically ERK and p38 kinases, which in turn induced IL-10 over-expression (218). To study signalling pathway activation and modulation in the presence of *C. jejuni* flagella, a phosphokinase array kit was used. Protein phosphorylation is a post-translational modification, which, in principle, triggers activation of the modified target. The array contains antibodies against a range of kinases embedded in the membrane. Following incubation of the membrane with the cell lysates, an anti-phospho-tyrosine antibody is used to detect the phosphorylation levels of these kinases. Levels of bound phosphoproteins can then be quantified by autoradiography. dTHP-1 cells were co-cultured with *C. jejuni* for 1h prior to cell lysis and analysis of kinase phosphorylation on radiography film (Figure 5.13A).

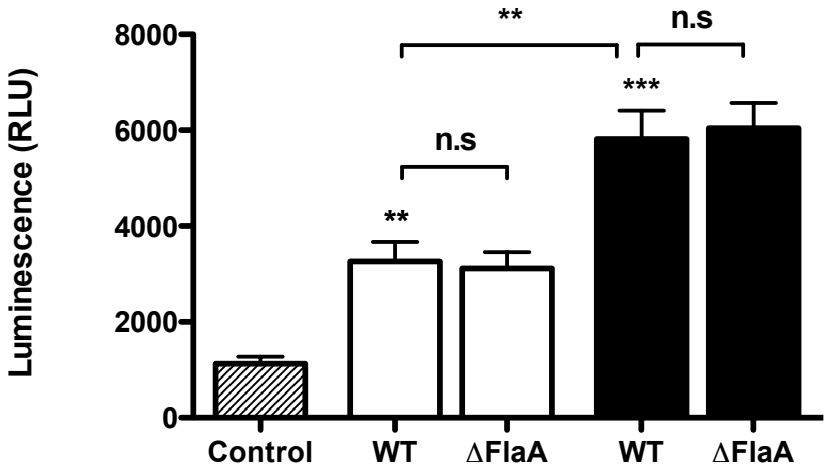

Figure 5.12. WT and *ΔflaA C. jejuni* mediate similar levels of NF-κB induction.
5x10^5 dTHP-1s transduced with a NF-κB-luciferase reporter plasmid were co-cultured with WT or *ΔFlaA C. jejuni* strain 11168H MOI 100 for 4h (white bar) or 8h (black bar). Luciferase activity was determined by spectrophotometry (450 nm). Values represent mean ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (**P<0.01, ***P<0.001).

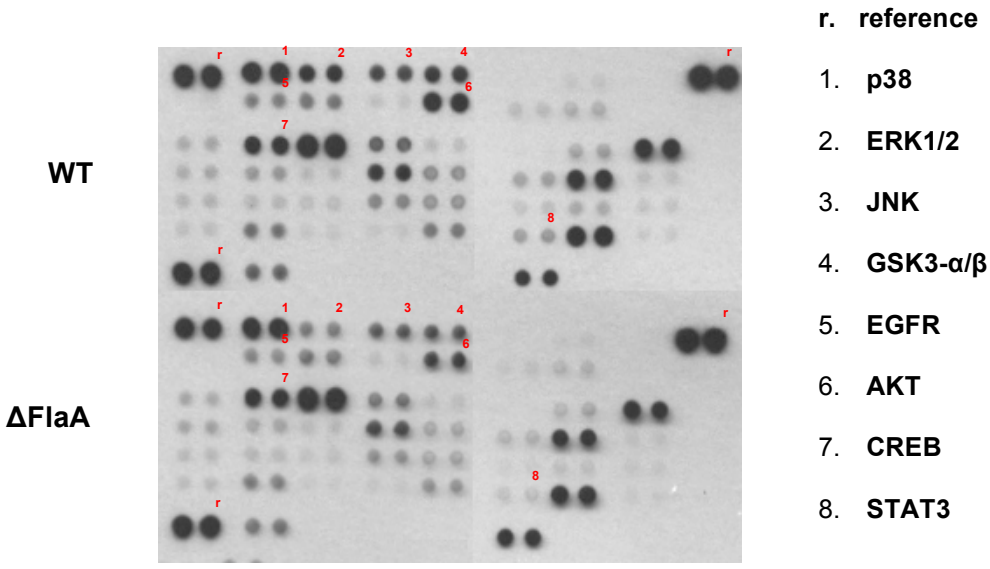

Figure 5.13A. WT and ΔFlaA *C. jejuni* induce differential dTHP-1 signalling as determined by phosphokinase array.

5x10^6 dTHP-1s were co-cultured with WT or *ΔFlaA C. jejuni* strain 11168H MOI 100. 1h post-infection, kinase phosphorylation was analysed using a Phospho-kinase Array kit by R&D. Image shown is a representative of three independent experiments analysed in duplicate.

The film was digitalised and signal intensity analysed by imaging software. Initially, data were ordered by pixel density to identify the most abundant phosphokinases in the dTHP-1 cell response to *C. jejuni* (Figure 5.13B; Mean Pixel Density). MAPKs were amongst the highest ranking, with p38 being the most abundant and ERK1/2 and JNK in the following ten places amongst 45 other proteins. High levels of phosphorylated Akt, RSK, c-Jun, GSK-3α/β, MSK-1 and CREB, all linked to MAPK-induced signalling were also detected. Other highly abundant phosphoproteins were HSP27, WNK1, STAT2, HSP60, PRAS40 and AMPKα2. To identify flagella-specific kinase activation, pixel density values between dTHP-1 cells co-cultured with WT and *ΔFlaA C. jejuni* were compared. Data was plotted as fold change in signal intensity between the two samples (Figure 5.13B; Fold Change). Strong differential induction in the presence of flagella was observed for ERK and JNK and less so for p38. Members of the STAT family of kinases, predominantly STAT3, also became significantly more phosphorylated. Finally, both Akt and GSK3-α/β appeared more phosphorylated in the presence of flagella. While each of the above metrics can be informative in terms of absolute values, we wanted to assess how the flagella of *C. jejuni* modulate kinase activation relative to the microorganism. Therefore, mean pixel density values were multiplied by fold change to identify flagella-specific protein hyper-activation relative to total protein activation (Figure 5.13B; Relative Change). With regards to MAPKs, our analysis revealed that ERK1/2 and p38, rather than JNK, become hyper-induced in the presence of *C. jejuni* flagella. Differential phosphorylation of Akt and GSK3-α/β was the next most significant in ranking, re-enforcing our earlier findings. Amongst other kinases that appear significantly more phosphorylated in the presence of flagella are RSK-1/2/3, CREB, HSP27, AMPK, WNK1 and c-Jun. Finally, we confirmed that the presence of *C. jejuni* flagella leads to AKT and MAPK hyper-activation by Western blotting (Figure 5.14).

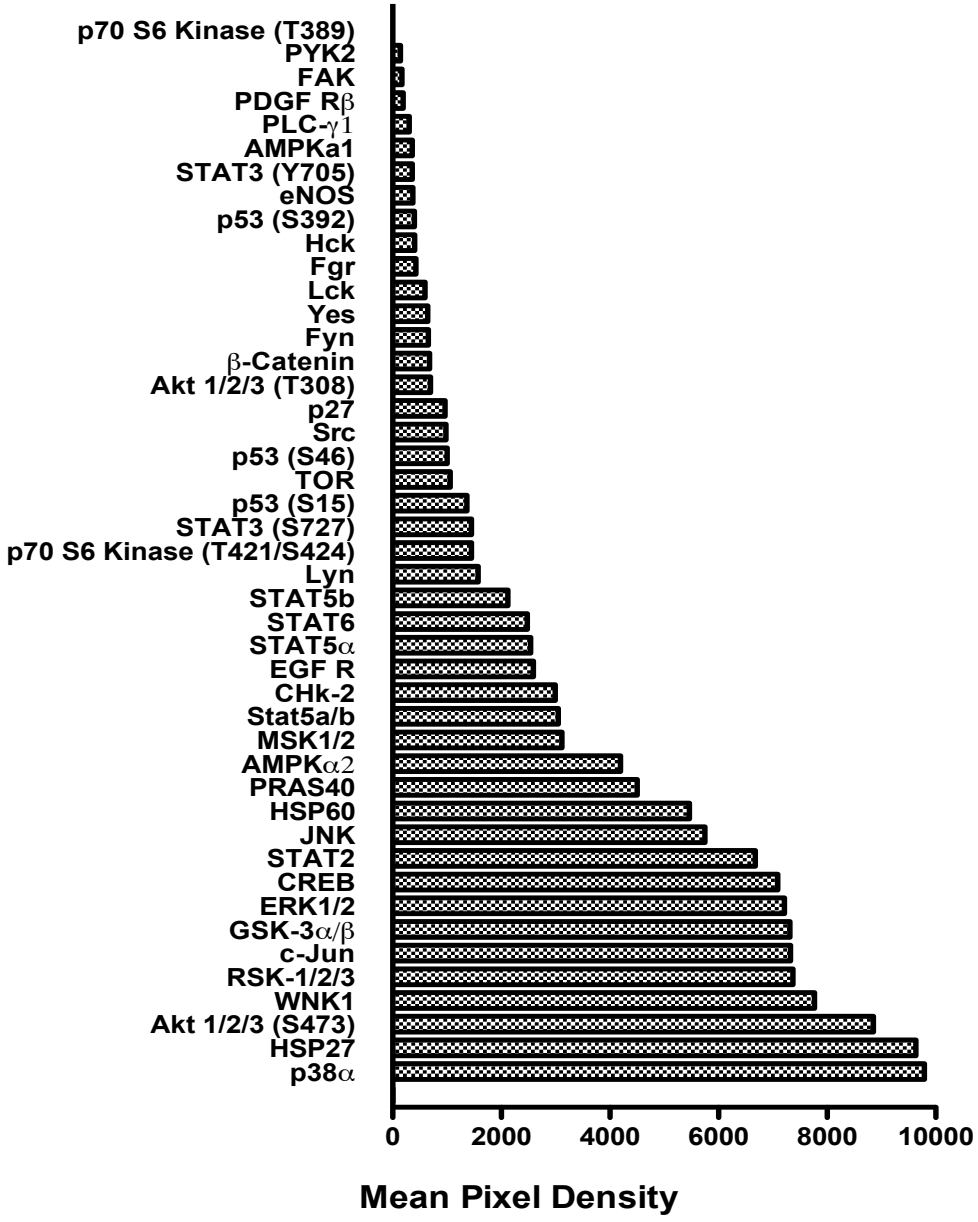

Figure 5.13B. WT and ΔFlaA *C. jejuni* induce differential dTHP-1 signalling as determined by phosphokinase array.

Pixel density analysis was performed using Gimp 2.8 software. Data shown is ordered by signal value (Mean Pixel Density) in WT *C. jejuni*-infected cells, signal value difference (Fold Change) in WT vs. *ΔflaA C. jejuni*-infected cells, or the product of their multiplication (Relative Change). Values are mean from three independent experiments analysed in duplicate.

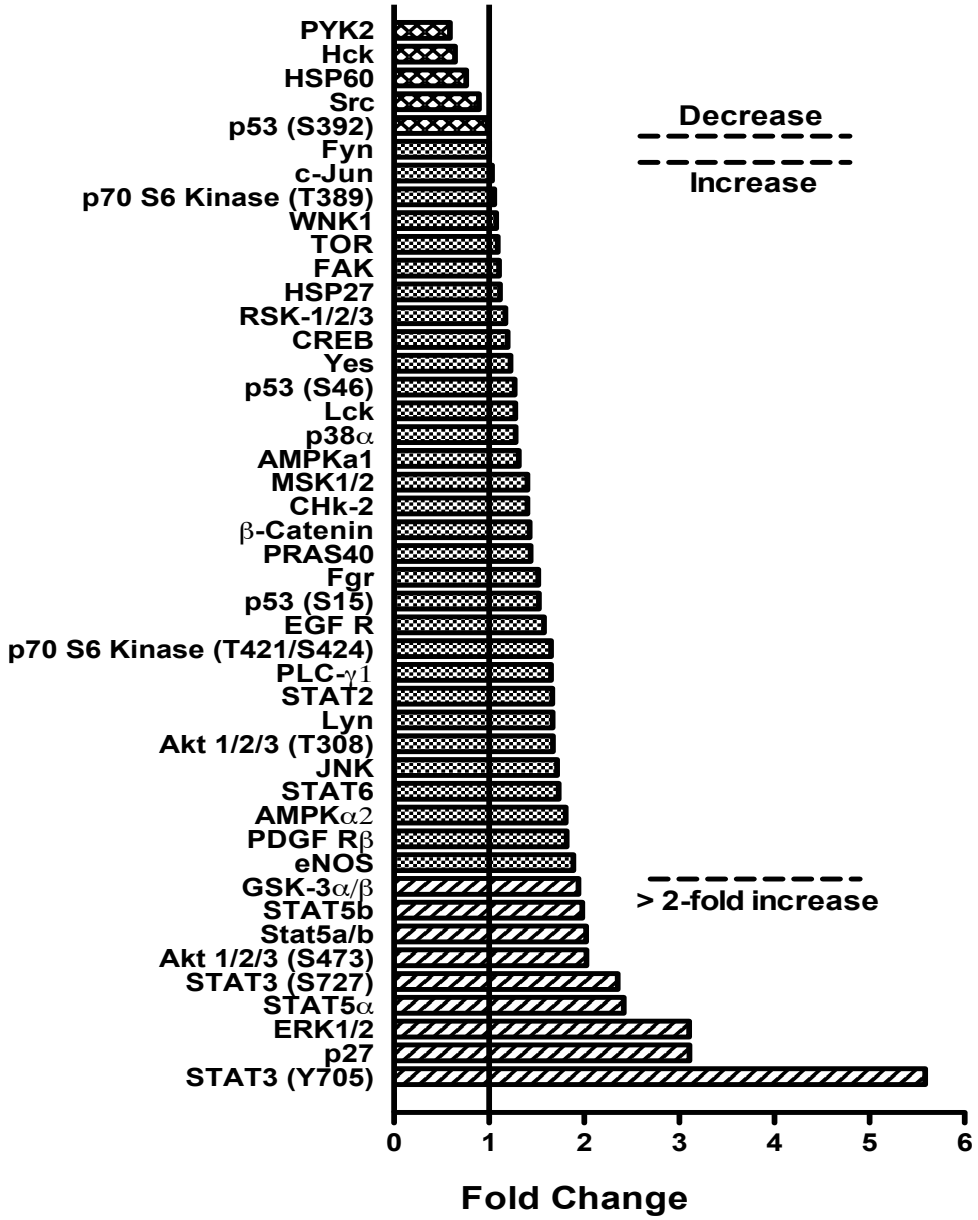

Figure 5.13B. WT and *ΔFlaA C. jejuni* induce differential dTHP-1 signalling as determined by phosphokinase array (continued).

Pixel density analysis was performed using Gimp 2.8 software. Data shown is ordered by signal value (Mean Pixel Density) in WT *C. jejuni*-infected cells, signal value difference (Fold Change) in WT vs. *ΔflaA C. jejuni*-infected cells, or the product of their multiplication (Relative Change). Values are mean from three independent experiments analysed in duplicate.

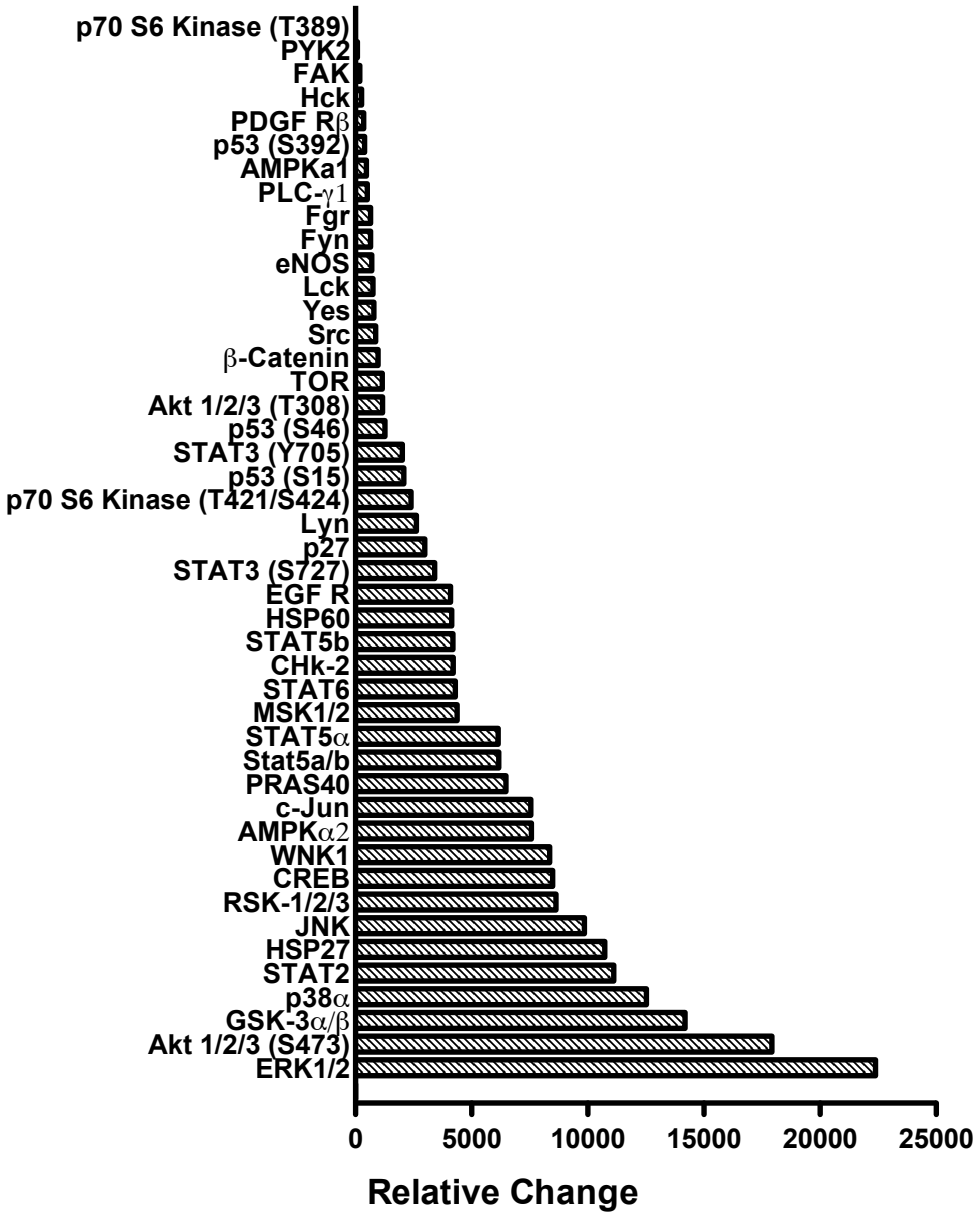

Figure 5.13B. WT and *ΔFlaA C. jejuni* induce differential dTHP-1 signalling as determined by phosphokinase array (continued).

Pixel density analysis was performed using Gimp 2.8 software. Data shown is ordered by signal value (Mean Pixel Density) in WT *C. jejuni*-infected cells, signal value difference (Fold Change) in WT vs. *ΔflaA C. jejuni*-infected cells, or the product of their multiplication (Relative Change). Values are mean from three independent experiments analysed in duplicate.

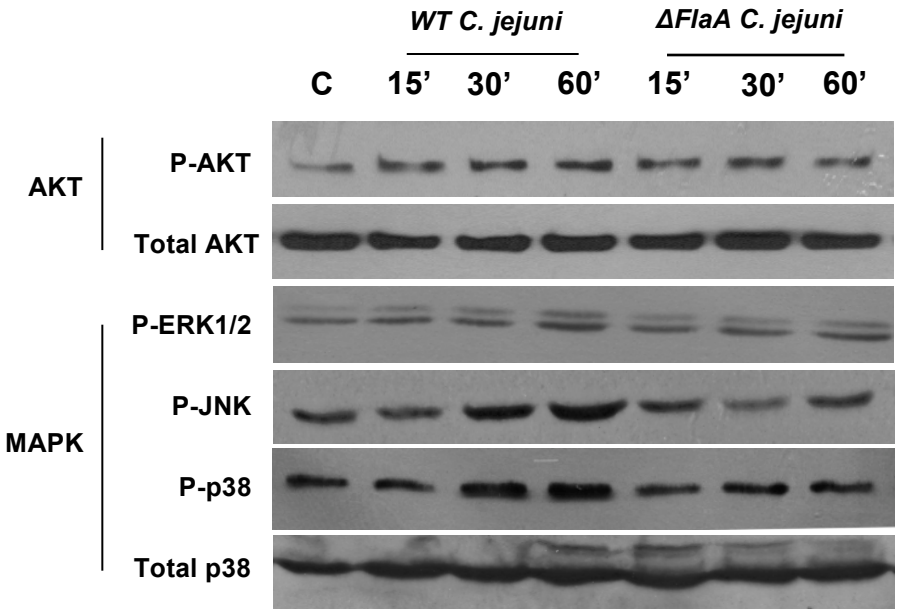

Figure 5.14. *C. jejuni* flagella lead to hyper-activation of AKT and MAPK signalling in dTHP-1 cells.

1x10^6 dTHP-1s were co-cultured with WT or *ΔFlaA C. jejuni* strain 11168H MOI 100. 1h post-infection, kinase phosphorylation was analysed at the indicated times by Western blot. Total p38 levels were used as loading control. Representative blot from two independent experiments is shown.

Our analysis of phosphokinase array data revealed four major signalling targets that become hyper-phosphorylated in the presence of *C. jejuni* flagella; MAPKs, AKT, STAT3 and GSK3. Importantly, all of these kinases are involved in the regulation of IL-10 expression, while both AKT and MAPKs have been linked to the immune response to *C. jejuni* (282), (368), (218). The role of these kinases in *C. jejuni*-mediated IL-10 induction was further assessed by the use of functional inhibitors. dTHP-1 cells were treated with ERK1/2-, JNK- and p38-specific inhibitors at increasing dosage prior to co-culture with *C. jejuni* strain 11168H. After 24h, IL-10 and TNFα protein levels were measured in the supernatant. Treatment with a low dose of ERK1/2 inhibitors led to a significant reduction in IL-10 (UO126; P=0.0013) but not TNFα cytokine levels (Figure 5.15). At a higher dose, we detected a decrease in the levels of both proteins, yet this was statistically more significant for IL-10 (UO126; p<0.0001, PD90859; p<0.0001) compared to TNFα (UO126; P=0.0033, PD90859; P=0.054). Inhibition of JNK only affected cytokine induction when a high dosage of the inhibitor was used. The effect appeared to have similar statistical significance for IL-10 (SP60025; P=0.0004) and TNFα (SP60025; P=0.0002). Again, both cytokines were affected by inhibition of p38 signalling with a low dose of inhibitors, although the reduction in secreted IL-10 (SB203580; P=0.0005, SB239023; p<0.0001) was statistically more significant compared to that of TNFα (SB203580; P=0.0149, SB239023; P=0.0069). The difference in cytokine production persisted when a high dosage of inhibitor was used, where again the decrease in IL-10 (SB203580; p<0.0001, SB239023; p<0.0001) was more pronounced than in TNFα (SB203580; P=0.0077, SB239023; P=0.0080). These findings reinforce the key role of MAPK signalling in the IL-10 to *C. jejuni* infection.

In a similar fashion, the role of AKT signalling in the IL-10 response to *C. jejuni* was investigated by treating dTHP-1 cells with inhibitors for AKT at a low and a high dose. Using low dose inhibitors, we observed decreased IL-10 and TNFα production after 24h, however reduction in secreted IL-10 (LY294002; P=0.0247, Wort; P=0.0026) was statistically more significant than in TNFα (LY294002; P=0.0132, Wort; P=0.0855) (Figure 5.16). Similarly, at a high dose, the reduction in IL-10 (LY294002; P=0.0021, Wort; P=0.0028) was again more significant in comparison to the reduction in TNFα signaling (LY294002; P=0.0025, Wort; P=0.0211). Next, using the Stat3 inhibitor stattic, we found both the IL-10 and TNFα response to *C. jejuni* to be Stat3-dependent, yet the reduction in IL-10 (Stattic; P=0.0011) was statistically more significant than in TNFα (Stattic; P=0.0023).

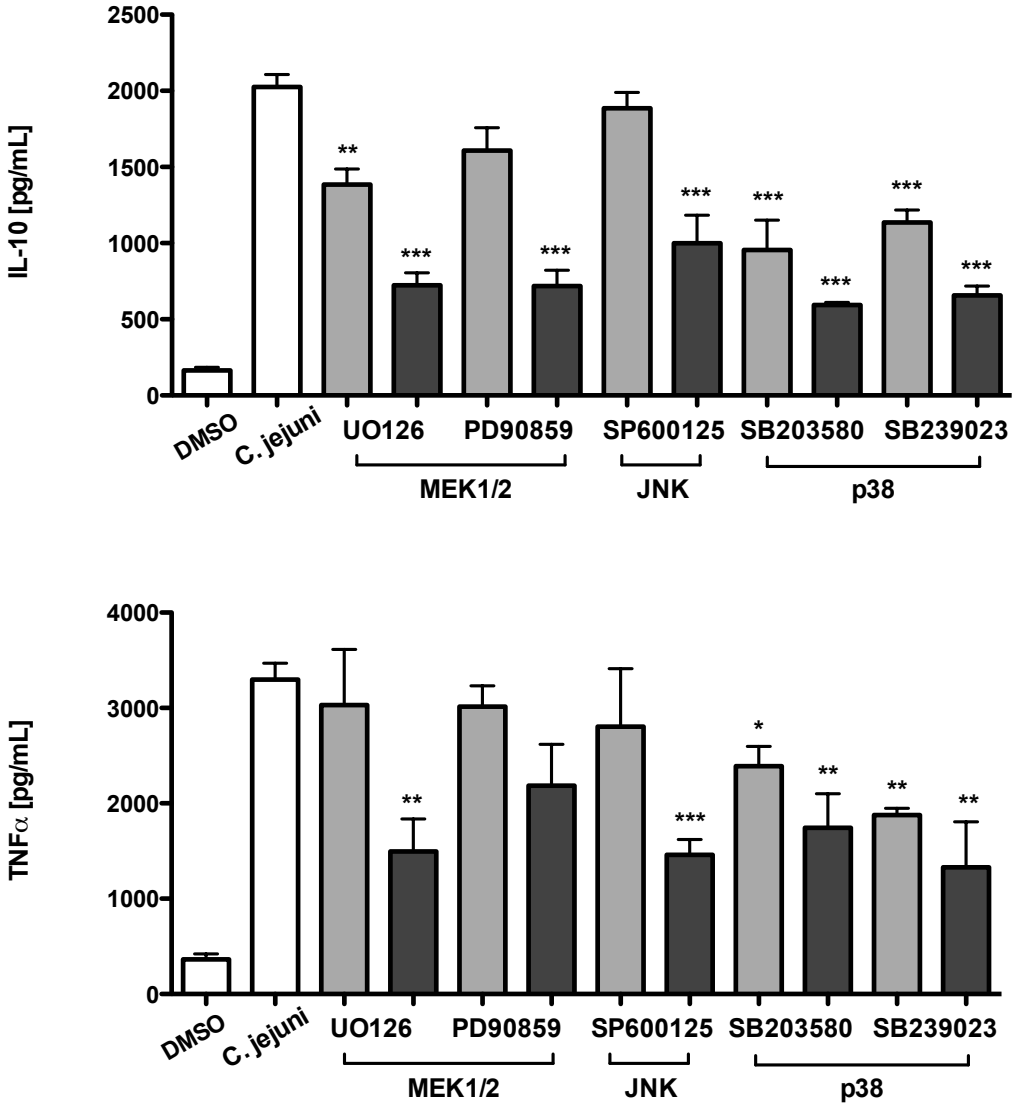

Figure 5.15. MAPKs play a critical role in *C. jejuni*-mediated IL-10 induction.

$5x10^5$ dTHP-1s were pre-treated with inhibitors of MAPK signalling; targeting MEK1/2 (U0126 & PD5090859), JNK (SP600125) or p38 (SB203580 & SB239063) at a concentration of 1µM (light bar) or 10µM (dark bar) (5µM and 50µM for PD5090859). 1h after treatment, cells were co-cultured with *C. jejuni* MOI 100 for 24h in the presence of inhibitors and the supernatants analysed for IL-10 and TNFα cytokines by ELISA. Values are mean ± SEM from three independent experiments performed and analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01, ***P<0.001).

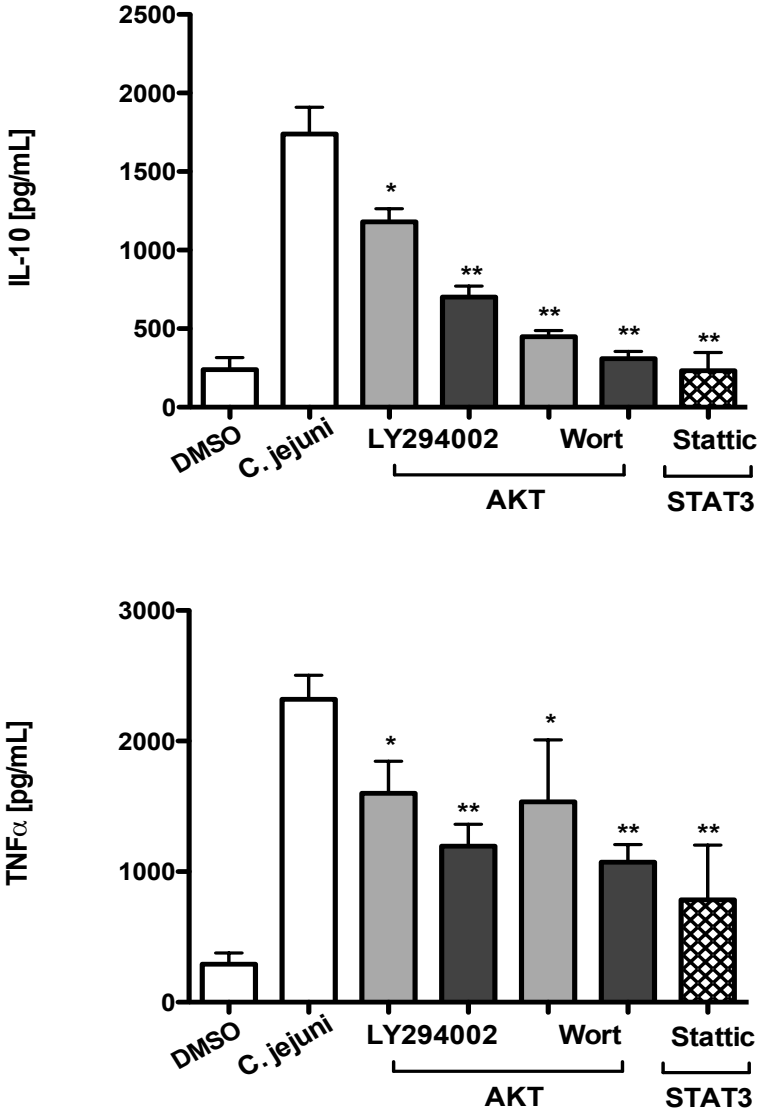

Figure 5.16. AKT and STAT3 signalling are important for *C. jejuni*-mediated IL-10 induction.

$5x10^5$ dTHP-1s were pre-treated with the AKT inhibitors LY294002 and wortmannin at a concentration of 10μM (light bar) or 50μM (dark bar) and the STAT3 inhibitor stattin (10μM). 1h after treatment, cells were co-cultured with *C. jejuni* MOI 100 for 24h in the presence of inhibitors and the supernatants analysed for IL-10 and TNFα cytokines by ELISA. Values are mean ± SEM from three independent experiments performed and analysed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

The final kinase identified by our phosphokinase study was GSK-3. This protein consists of an α and β subunit (GSK3-α/β) and acts as a negative regulator of IL-10 expression when de-phosphorylated. Phosphorylation of the β subunit leads to the inactivation of GSK3 and subsequent removal of IL-10 expression blockage. LiCl was used to inhibit the phosphorylation of GSK-3β in dTHP-1 cells co-cultured with *C. jejuni* and IL-10 cytokine levels were assessed after 24h. While treatment with LiCl alone, triggered induction of IL-10, this was not statistically significant (Figure 5.17). A similar enhancement of the IL-10 response was observed in cells co-cultured with WT *C. jejuni* strain 11168H, which did not reach statistical significance. However, in dTHP-1 cells co-cultured with *ΔFlaA C. jejuni* strain 11168H, IL-10 production was significantly higher in the presence of LiCl (P=0.0374). Therefore, flagella-induced phosphorylation of GSK3-β is a critical step in the differential induction of IL-10 by WT *C. jejuni*, as it deactivates suppression of the cytokine. In conclusion, we have been able to verify the involvement of those kinases identified through our phosphokinase array study (MAPK, AKT, Stat3 and GSK3) (see Figure 5.11A) in the immune response to *C. jejuni* and exhibit their importance in the pathogen-driven induction of IL-10.

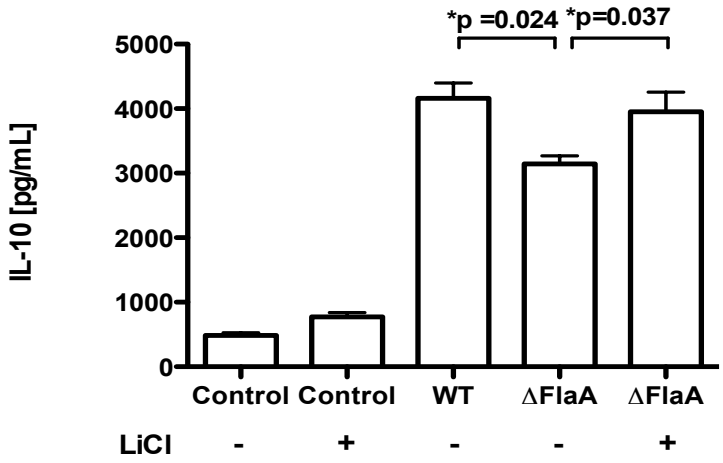

Figure 5.17. Inhibition of GSK-3β phosphorylation restores IL-10 secretion in *C. jejuni* FlaA mutant.

$5x10^5$ dTHP-1s were pre-treated with 1mM LiCl to inhibit GSK-3β phosphorylation. 1h after treatment, cells were co-cultured with *C. jejuni* MOI 100 for 24h in the presence of LiCl and the supernatants analysed for IL-10 cytokine by ELISA. Values are mean ± SEM from three independent experiments analysed in duplicate. Statistical analysis by paired t-test (*P<0.05).

5.2.3 *C. jejuni* flagella induce EGFR activation

Our work this far has outlined the differential activation of signalling cascades leading to induction of IL-10 in cells co-cultured with WT vs. the aflagellate *ΔFlaA* mutant of *C. jejuni* strain 11168H. In order to investigate the effects of *C. jejuni* flagella in greater detail, flagella were isolated and coupled to carboxyl-modified beads. Based on our findings of TLR-independent enhancement of AKT and MAPK signalling by *C. jejuni* flagella, we identified EGFR, a signalling factor upstream of both these cascades, as a likely mediator of flagellum-induced signalling. Phosphorylation of EGFR was 1.7x higher in the presence of flagella in dTHP-1 cells co-cultured with *C. jejuni* strain 11168H (Figure 5.13B). Therefore, we hypothesised that the flagellum of *C. jejuni* would activate EGFR. dTHP-1 cells were treated with WT and *ΔFlaA C. jejuni* strain 11168H, as well as flagella coupled to beads. Treatment with uncoupled beads and *C. jejuni* LOS were used as experimental controls. EGF-R phosphorylation was analysed by flow cytometry using an APC-conjugated anti-human phospho-EGF-R antibody (Figure 5.18A). According to our results, WT but not *ΔFlaA C. jejuni* was able to induce EGFR phosphorylation at statistically significant levels (P=0.0394) (Figure 5.18B). Interestingly, flagella alone were able to activate EGF-R in treated dTHP-1 cells (P=0.0101). Although an increase in the number of phospho-EGFR-positive cells was observed following treatment with LOS (Figure 5.18A), that shift was not statistically significant (Figure 5.18B).

To test whether *C. jejuni* flagella-induced activation of EGFR would sufficiently compensate for the diminished IL-10 response to the aflagellate pathogen, dTHP-1 cells were co-cultured with WT and *ΔFlaA* of *C. jejuni* 11168 alone or in the presence of flagella-coupled beads. Isolated *C. jejuni* flagella were not able to induce IL-10 production or rescue IL-10 induction in response to *ΔFlaA C. jejuni* (Figure 5.19). We

reasoned that the plurality of virulence factors in the pathogen might confound any signalling driven by the isolated flagella and used purified LOS of *C. jejuni* to gain more insight. While LOS-mediated induction of IL-10 was marginally enhanced in the presence of isolated flagella, the difference was not statistically significant. Therefore, while treatment of dTHP-1 cells with *C. jejuni* flagella led to EGF-R activation, it cannot induce or enhance production of IL-10 on its own.

5.5 Discussion

Expanding on previous work from our group, we investigated the signalling mechanisms involved in flagellum-mediated enhancement of the host IL-10 response to *C. jejuni*. While previous work had been carried out in murine cells, we focused on innate immune cells of human origin where *C. jejuni* is an opportunistic pathogen. First, a *C. jejuni* infection model was established based on the co-culture of the pathogen with monocyte-derived DCs (Figures 5.2). As IL-10 is a late response gene (338), significant cytokine levels were only detected after 24h (Figure 5.3). The cytokine response of primary DCs to *C. jejuni* was comparable to that of Stephenson et al. (218) and similar to BMDCs findings from previous studies (231). Subsequently, we employed the flagellate *ΔFlaA* mutant of *C. jejuni* to investigate the mechanisms of flagellum-mediated IL-10 induction. As we show in Figure 5.4, the flagellum of *C. jejuni*, amongst other proteobacteria, has evolved to evade TLR5 recognition (217), which may contribute to host evasion by avoiding activation of APCs (369). In conjunction with the ability of *C. jejuni* flagella to enhance IL-10 production in these cells (218), a rather fetching hypothesis forms around the tolerogenic role of the flagella structure. Similar to our earlier findings in BMDCs (218), the absence of flagella led to a diminished IL-10 response, while TNFα levels were unaffected (Figure 5.5).

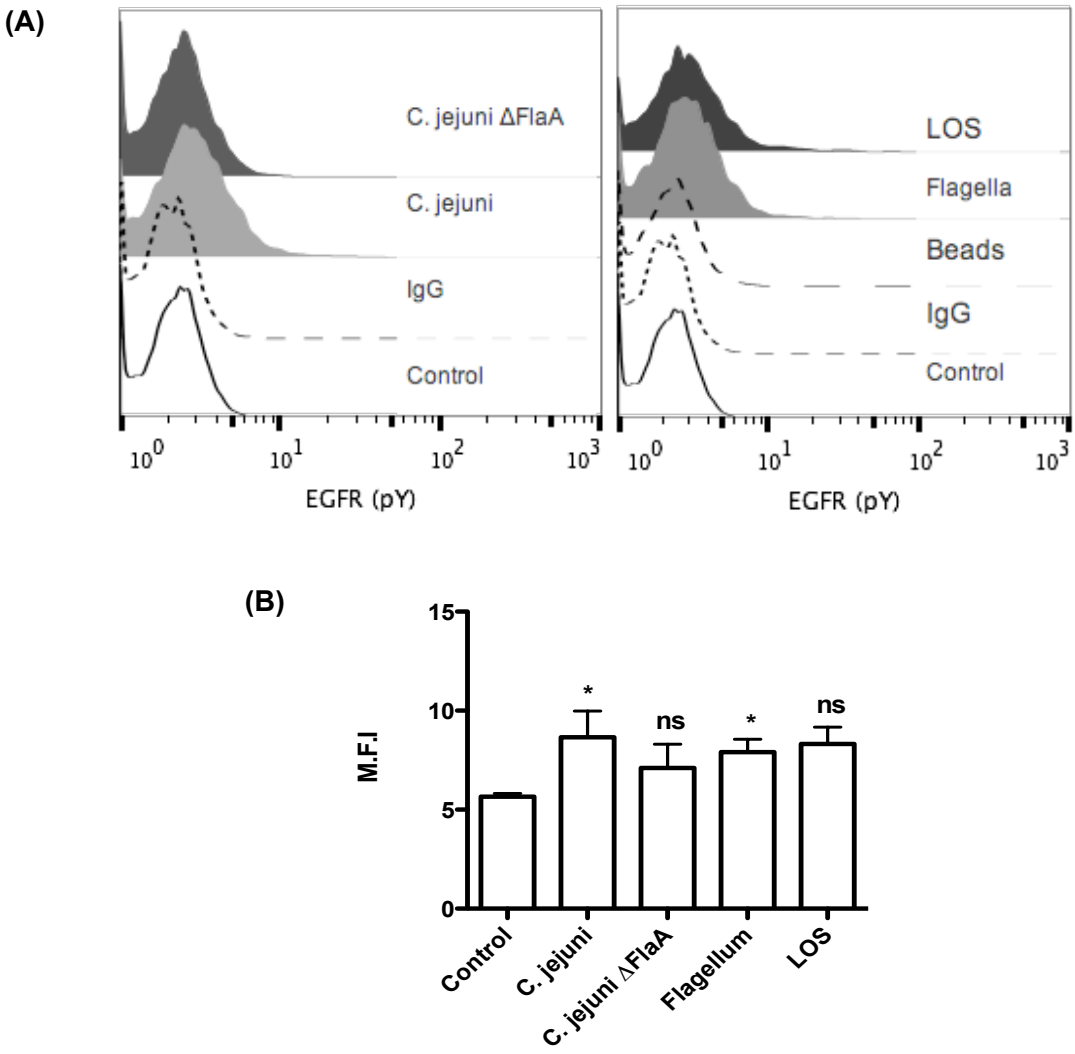

Figure 5.18. *C. jejuni* flagellum induces EGFR phosphorylation.

5x10^5 dTHP-1s were co-cultured with (A) WT or *ΔFlaA C. jejuni* (left panel), beads or flagella-coated beads (10µl/ml) and *C. jejuni* LOS (1ng/ml) (right panel). 1h post-stimulation, phosphorylated EGFR was detected by intracellular staining and analysed by flow cytometry. Histograms shown are representative of three independent experiments performed in duplicate. (B) Chart of mean fluorescence intensity (M.F.I) values expressed as Mean ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (WT *C. jejuni*; P=0.0394 , Flagellum P=0.0101).

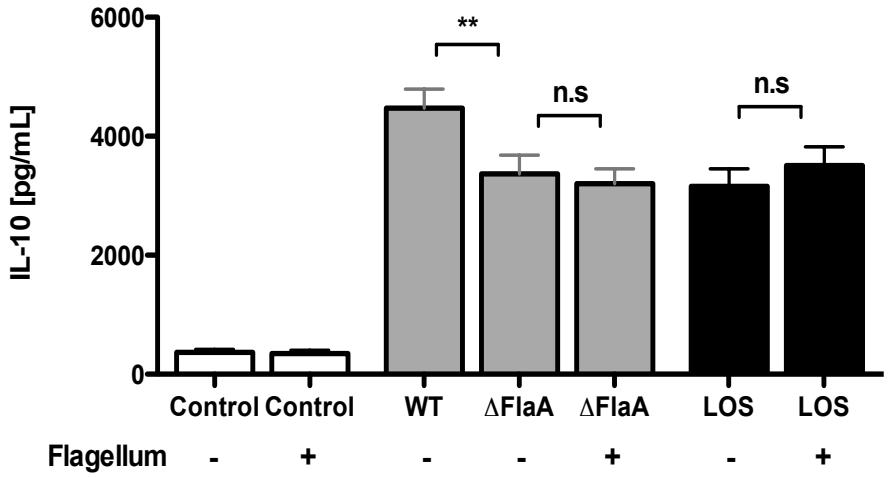

Figure 5.19. Effect of isolated flagellum on the IL-10 response to *C. jejuni*.

$5x10^5$ dTHP-1s were co-cultured with WT or *ΔFlaA C. jejuni* MOI 100 and 1ng/ml LOS alone or in the presence of 10µl/ml *C. jejuni* flagellum-coated beads. 24h post-stimulation, IL-10 cytokine was analysed in the supernatants by ELISA. Values are mean ± SEM from three independent experiments analysed in duplicate. Statistical analysis by paired t-test (**P=0.0037).

This was consistent in monocyte-derived DCs and macrophages; with both M1- and M2-type macrophages exhibiting flagella-dependent enhancement of IL-10 production. The effect was significantly more pronounced in tolerogenic M2-type macrophages where IL-10 transcription is enhanced via epigenetic changes during differentiation (370), yet evident in all three cell types. Differential IL-10 induction in the absence of flagella was also observed in dTHP-1 cells (Figure 5.6). The effect was independent of bacterial motility as bacterial adherence (Figure 5.7) and survival (Figure 5.9) were unaffected in the absence of flagella. Whereas the flagella are required for the invasion of intestinal immune cells (371), dTHP-1 cells phagocytose bacteria rapidly (within 30min) (Figure 5.8). It is worth noting that the FlaA gene contains a variable region and such variability may affect the interaction of the flagella with the Siglec-10 receptor as well as the induction of IL-10. A comparison between strains with different FlaA variants would contribute greatly to our study.

The phosphokinase array employed to delineate signalling events upstream of IL-10 transcription, revealed the activation of two main pathways by *C. jejuni*; the MAPK and the PI3K signalling pathways (Figure 5.11B). All three members of the MAPK family were activated by *C. jejuni*, as others have previously reported (245). Akt, a kinase of the PI3K signalling cascade, was also highly phosphorylated. PI3K signalling was recently found to contribute to *C. jejuni*-induced colitis in mice (282). Both ERK and p38 signalling pathways have been implicated in the induction of IL-10 (367) and ERK activation is required for optimal IL-10 induction in macrophages and DCs (338). As expected, downstream members of these two pathways also became phosphorylated. In the MAPK pathway, the chaperone protein HSP27 undergoes ERK-dependent phosphorylation (372) and the transcription factor c-Jun is a target of JNK (273). In the PI3K cascade, WNK1 mediates Akt-dependent signalling (373). Interestingly, some of

these kinases are known regulators of IL-10 expression; as discussed earlier GSK3-β acts as a repressor of IL-10 and the transcription factor CREB controls IL-10 expression in macrophages (338). Downstream of both ERK and p38, the RSK-1/2/3 complex drives epigenetic modifications in the IL-10 promoter by phosphorylating H3 (S10) to facilitate transcription (374). Overall, the strong activation of ERK, p38 and Akt reinforces our hypothesis that *C. jejuni* induces IL-10 production in macrophages as an immune evasion mechanism. Finally, C.jejuni induced phosphorylation of AMPK-2α, a member of the AMP-activated protein kinase (AMPK) signalling pathway, which becomes activated when intracellular ATP levels fall (375). ATP production decreases as cells switch from metabolic processes to defence functions.

By comparing WT to *ΔFlaA C. jejuni*, we detected differential activation of signalling pathways in co-cultured cells in the presence of the flagella. Phosphorylation of STAT3 and STAT5, two ERK-dependent transcription factors that bind the IL-10 locus (376), was more than 2-fold higher. Both MAPK and PI3K signalling was enhanced in cells co-cultured with flagellated WT *C. jejuni*. While ERK and JNK showed higher differential activation compared to p38, relative induction was higher for ERK and p38 (Figure 5.11B). When activation of MAPKs was analysed by western blotting, ERK and p38 rather than JNK, appeared to become hyper-activated in the presence of flagella (Figure 5.12). The importance of both signalling pathways in *C. jejuni*-mediated IL-10 induction was confirmed by inhibitor studies (Figure 5.13, Figure 5.14 and Figure 5.15). MAPK activation by *C. jejuni* is TLR-mediated (250), (368) and drives the induction of IL-10 as well as pro-inflammatory mediators (377), (378). Nevertheless, inhibition of either ERK or p38 activation leads to a reduction, but not abrogation, of IL-10 expression (367), which suggests that these two pathways might cooperate in TLR-induced IL-10 production. Likewise, TLR signalling leads to activation of Akt (379),

although the mechanism of *C. jejuni*-mediated PI3K activation has not been studied in detail.

In the case of *C. jejuni*, however, hyper-induction of MAPK and Akt signalling by the flagella is TLR5-independent. Using isolated flagella coupled to beads we were able to show that *C. jejuni* flagella alone is able to activate EGFR (Figure 5.16). Due to study limitations, the purity of the flagella preparation was not assessed for the presence of contaminants such as LOS. As the receptor sits upstream of both signalling cascades (273), we propose that flagella-mediated EGFR activation enhances MAPK and Akt signalling. In support of our hypothesis, Pi3K activation by *Pseudomonas aeruginosa* is dependent on EGFR activation and, furthermore, isolated flagella were shown to activate EGFR (380). Yet, amalgamation of more signals may be required for IL-10 hyper-induction, as co-stimulation with flagella-coupled beads was not sufficient to restore cytokine levels. IL-10 gene expression requires rapid transient modifications of the promoter augmented by transcription factor recruitment in a remarkably time-limited sequence (381), therefore the addition of isolated flagella may not replicate the kinetics of WT *C. jejuni* interaction with the cells. In work published by our group and discussed in detail in Chapter 6, we showed that *C. jejuni* flagella-specific binding to the Siglec10 receptor led to an increase in IL-10 production (218). To our knowledge, this is one of the first TLR5-independent flagellum interactions reported, so the involvement of other receptors in flagellum recognition will need to be investigated in more depth.

While the role of IL-10 in the interaction of *C. jejuni* with host immunity remains unclear, another interesting link between *C. jejuni* and IL-10 expression has emerged. In 2006, Manfield *et al.* demonstrated *C. jejuni*-induced colitis in IL-10-/- mice at a higher rate than the lack of other immune elements such as TLRs and NODs (382). These gnobiotic IL-10 deficient mice lack the anti-inflammatory signalling to counter-act the

onset of colitis and have since been used in a number of studies of *C. jejuni*-induced enteritis (71), (383). Yet, while the role of intestinal IL-10 is pivotal for the onset of *C. jejuni*-induced enteritis, its role in *C. jejuni* pathogenesis is not yet fully understood.

Chapter 6

Campylobacter jejuni interaction with Siglec receptors

6.1 Introduction

As shown in Chapter 5 the flagellum of *C. jejuni* modulates the IL-10 response to the pathogen. We therefore went on to investigate possible receptors involved in flagellum recognition. We have shown that glycosylated flagellum was able to modulate IL-10 expression, suggesting that host glycan receptors may play a role. An emerging paradigm suggests that microbes can modulate host IL-10 expression via engagement of glycan receptors, for example *Mycobacterium tuberculosis* and *Candida albicans* engage DC-SIGN on DCs (384),(385). Studies have previously reported that strains of *C. jejuni* are recognised by members of the Siglec receptor family (262). More recently, our group showed that Siglec-10 specifically binds to the flagellum of *C. jejuni* (218). Siglecs have emerged as important players in the regulation of many cellular processes including endocytosis, apoptosis, cellular activation, and proliferation (253). Siglecs bind sialylated structures via a terminal V-set Ig domain. They exhibit varying specificities for the linkage of the sialic acid (SA) and the underlying glycan structure which can be present on both the host cell and on microbes, thus promoting both cis- and trans-interactions (386).

In general, Siglecs show low affinity for the SA N-acetylneuraminic acid (Neu5Ac) α2,3 and α2,6 linkages to galactose (Neu5Acα2–3Gal and Neu5Acα2–6Gal) that are commonly found as terminal sequences on glycans of glycoproteins and glycolipids of most mammalian cells (387). Yet some pathogenic microorganisms, including *C. jejuni*, have evolved the ability to synthesise SAs that serve as molecular mimics of the host cell surface in order to evade immune activation (388). *C. jejuni* can express monosialylated and disialylated LOS with a2,3- or a2,3/2,8-linked SA residues, respectively. LOS sialylation can affect phagocytosis and the cytokine response to *C. jejuni* (389). It has also been associated with increased severity of gastroenteritis(390), as well as with the development of Guillain-Barré syndrome (391).

For the largest part, research on the interaction of *C. jejuni* with Siglecs has focused on deciphering the link between *C. jejuni* infection and the onset of Guillain-Barré. Molecular mimicry between *C. jejuni* sialylated LOS and human nerve gangliosides can trigger the production of cross-reactive antibodies leading to induction of Guillain-Barré(392). Strains of *C. jejuni* associated with Guillain-Barré contain disialylated LOS that is recognised by Siglec-7 (262),(393) and monosialylated LOS recognized by sialoadhesin (Sn; also known as Siglec-1) (263),(394). Our interest lies in the enteric pathology of *C. jejuni* and, hence, the reference *C. jejuni* strain 11168, a clinical isolate associated with enteritis, was used. *C. jejuni* 11168 presents GM1a and GM2 ganglioside mimicry and therefore contains monosialylated α2,3-linked Neu5Ac on its surface. Enteritis-associated strains of *C. jejuni* with sialylated LOS are recognised by Sn (263), although no link to disease onset exists. The 11168 strain used in this study can bind to Sn (395), but not Siglec-7 (393). Despite the extensive use of *C. jejuni* 11168 in research, very little is known regarding its interaction with other members of the Siglec family.

6.1.2 Siglec-10 binding to *C. jejuni* flagellum

A novel interaction between *C. jejuni* and a member of the Siglec family was recently uncovered by our team. The Siglec-10 receptor was shown to bind to structures on the flagellum of *C. jejuni* 11168 (218). While the surface of *C. jejuni* contains SA structures, the flagellum proteins of *C. jejuni* are O-linked glycosylated with Sia-like structures, derivatives of pseudaminic acid (Pse), and sometimes legionaminic acid (Leg), moieties. These moieties are important for flagellum assembly and colonisation in chickens (216),(213). In particular, the Pse5Ac7Am moiety, which is structurally related

to Neu5Ac, appeared to be critical. Furthermore, the interaction between *C. jejuni* and Siglec-10 receptor was independent of the Neu5Ac. In Chapter 5, we reported on the capacity of the flagellum to modulate IL-10 production in response to *C. jejuni*. Therefore, we became interested in the functional implications of flagellum recognition by Siglec-10 and, more specifically, in whether flagellum binding to Siglec-10 could affect IL-10 induction by *C. jejuni* 11168.

6.2 Results

6.2.1 Role of Siglec-10 in *C. jejuni* flagellum-mediated IL-10 induction

As discussed in Chapter 5, *C. jejuni* flagellum led to enhancement of IL-10 production in DCs and macrophages. Siglec-10 expression has only previously been reported in human monocytes and DCs, but not macrophages(256). We therefore differentiated primary human monocytes into both M1- and M2-type macrophages and analysed them for expression of Siglec-10. Both cell types were positive for Siglec-10 expression (Figure 6.1) although levels amongst donors were variable. Furthermore, pre-monocytic THP-1 cells and PMA-differentiated THP-1 (dTHP-1) cells expressed Siglec-10 (Figure 6.2). We observed increased Siglec-10 expression following PMA maturation of THP-1 cells, indicating induction of the receptor in mature macrophages.

To study the potential link between Siglec-10 and *C. jejuni* flagellum-mediated IL-10 induction, we over-expressed the receptor in the THP-1 cell line. Cells were transduced at two different MOIs (MOI 1 and MOI 10) and sorted according to Siglec-10 expression to yield two distinct cell lines: Siglec-10-Low and Siglec-10-High, expressing lower and higher levels of the receptor respectively (Figure 6.3). Following PMA treatment, the cells were co-cultured with WT and *ΔFlaA C. jejuni* MOI 100 for 24h (Figure 6.4). Besides IL-10, we analysed the production of TNFα, which is unaffected by the flagellum (see Chapter 5), and IL-12 which shares regulatory pathways with and is inhibited by IL-10(396). GFP-transduced cells were also used as a control for the effects of transduction in cytokine production. We observed an increase in the flagellum-mediated enhancement of IL-10 in low ($P=0.021$) or high ($p<0.0001$) Siglec-10 over-expressing cells compared to WT THP-1 cells ($P=0.032$) (Figure 6.4). The effect was flagellum-specific as there was no difference in IL-10 production amongst

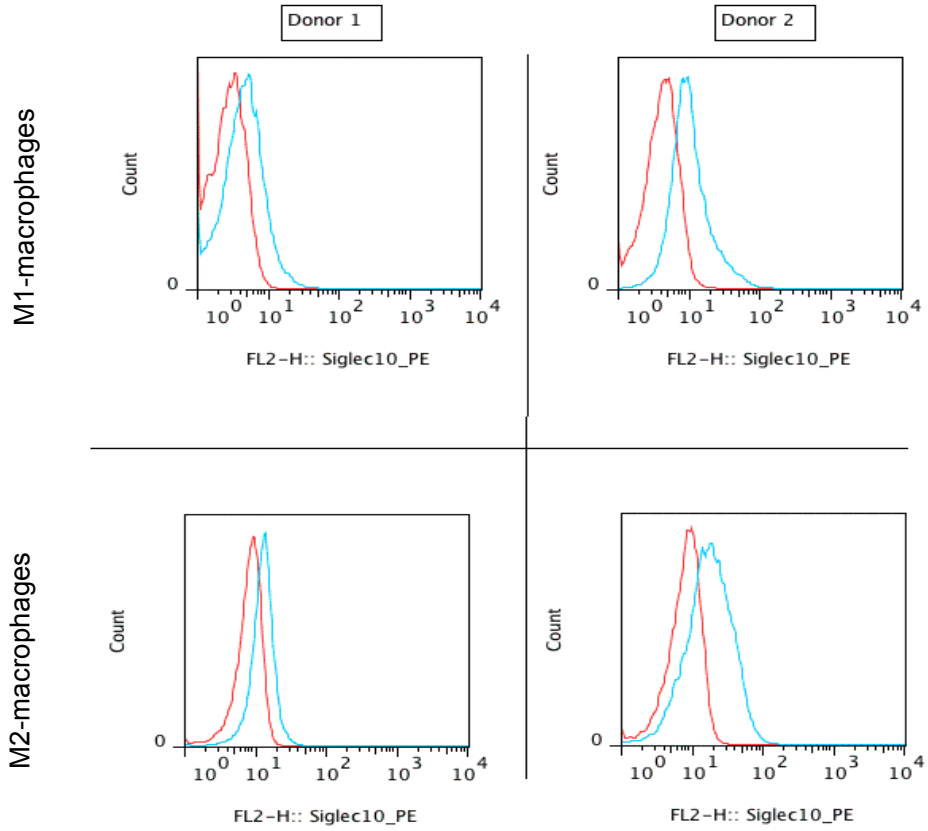

Figure 6.1. Siglec-10 expression on human MDMs.

$5x10^5$ primary MDMs from four donors were cultured in media with 20ng/ml GM-CSF for M1-type or 20ng/ml M-CSF for M2-type differentiation for 5d, followed by 24h in fresh media. On day 6, cells were immunostained with a conjugated anti-Siglec-10 antibody (blue line) or IgG control (red line) and analysed by flow cytometry (representative histograms from two individual donors shown).

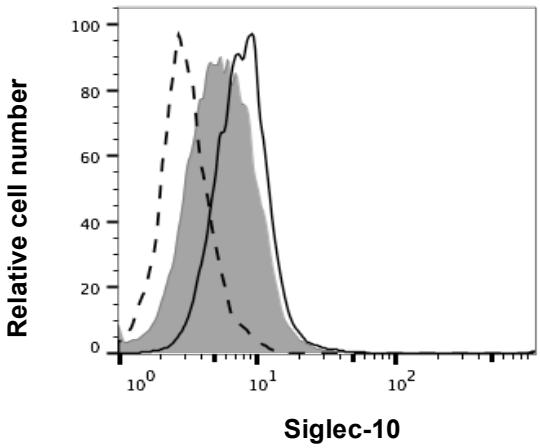

Figure 6.2. Siglec-10 is expressed on monocytic THP-1 and dTHP-1 cells.
Siglec-10 expression on THP-1 and dTHP-1 cells was assessed before (solid grey line) or after (black line) treatment with 10ng/ml PMA for 24h by flow cytometry. IgG1 isotype staining (dashed line) served as control. Histogram is representative of three independent experiments.

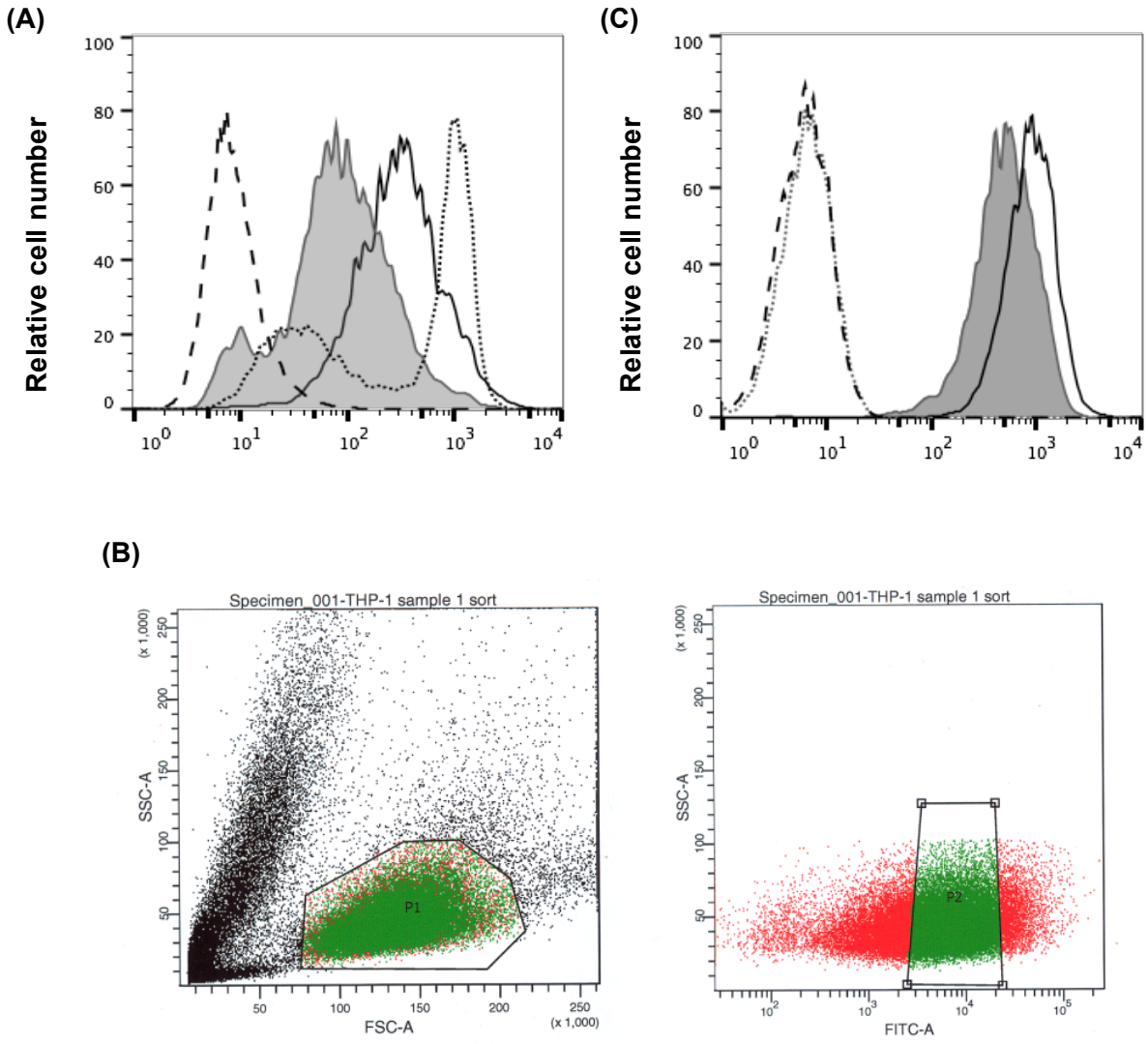

Figure 6.3. Siglec-10 receptor overexpression in THP-1 cells.

(A) THP-1 cells were left untransduced (dashed line) or transduced with eGFP MOI 10 (dotted line), Siglec-10-GFP MOI 1 (solid gray line) or MOI 10 (black line) vectors. After 72h, GFP-positive cells were quantified by flow-cytometry to assess transduction efficiency. (B) Cell populations were sorted by FACS based on GFP expression (representative dotplot from Siglec-10-High selection is shown). (C) Siglec-10 expression was analysed by flow cytometry. Histogram is representative of two independent experiments.

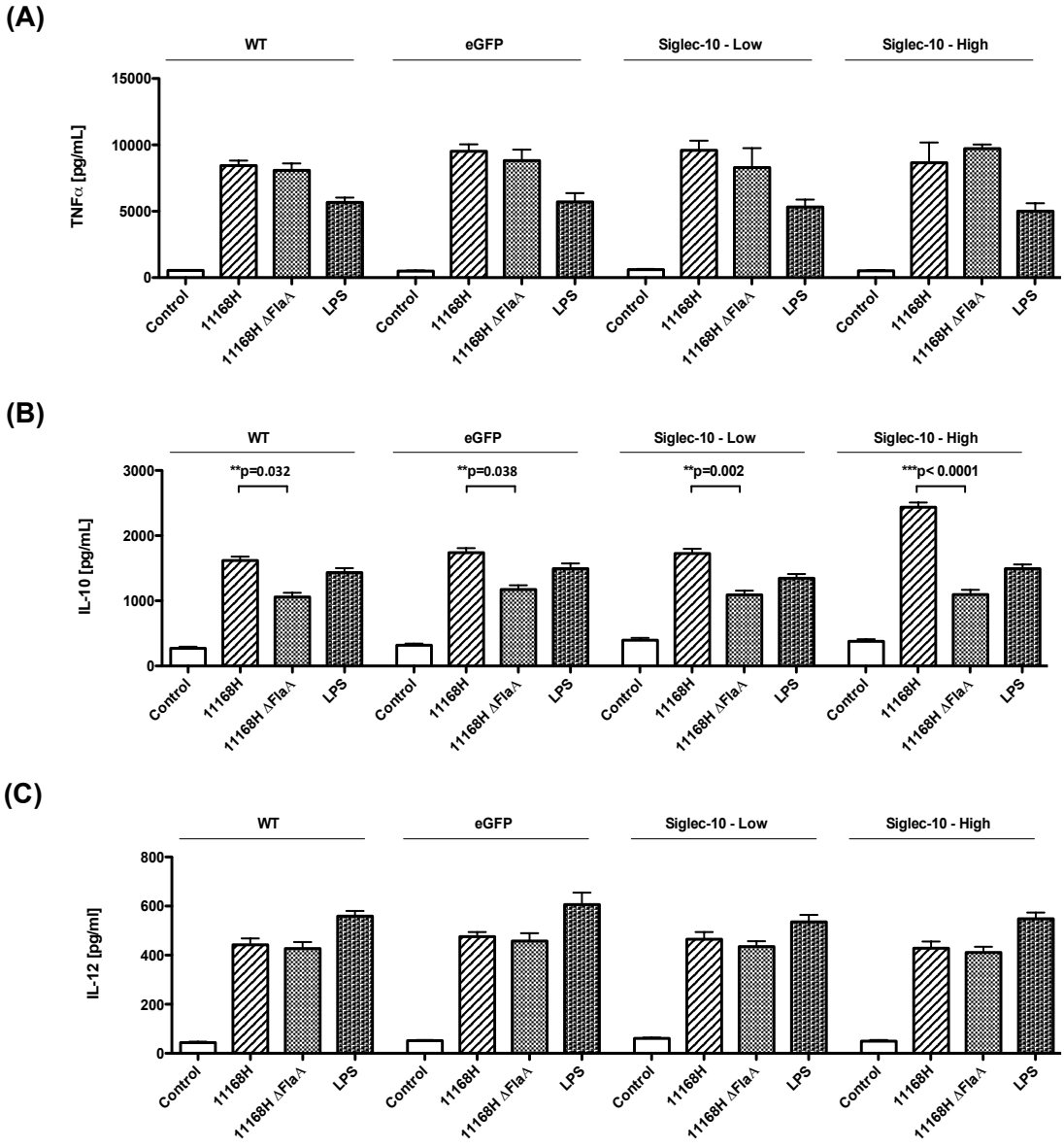

Figure 6.4. Siglec-10 receptor over-expression promotes IL-10 expression in response to *C. jejuni* flagella.

Untransduced, eGFP-transduced, Siglec-10-Low or Siglec-10-High dTHP-1 cells were co-cultured with WT or *ΔFlaA C. jejuni* MOI 100. 10ng/ml LPS stimulation was used as a control. 24h post-infection, (A) TNFα, (B) IL-10 and (C) IL-12 cytokines were analysed in the supernatants by ELISA. Values are means ± SEM from three independent experiments analysed in duplicate. Statistical analysis by paired t-test (**P<0.01, ***P<0.001).

cells co-cultured with *ΔFlaA C. jejuni*. TNFα and IL-12 levels were not significantly different between WT and transduced cells and remained unaltered in the absence of *C. jejuni* flagellum. Overall, our findings suggest that Siglec-10 can mediate flagellum-specific enhancement of the macrophage IL-10 response to *C. jejuni*.

6.2.2 Immune receptor activation by *C. jejuni*

Based on the interaction of *C. jejuni* and Siglec-10, we went on to determine potential interactions of the pathogen with other members of the Siglec family. Siglec-7, Siglec-9 and Siglec-10 have been shown to bind *C. jejuni* serostrain HS:19 (262), although only the interaction with Siglec-7 was studied in depth. We used an Immunoreceptor Phosphorylation Array kit by R&D to detect phosphorylation of multiple immunoreceptors, including Siglecs, in dTHP-1 cells co-cultured with WT *C. jejuni* 11168 (Figure 6.5). Our analysis revealed phosphorylation of multiple Siglec receptors in the presence of *C. jejuni*: Siglec-3 (also known as CD33), Siglec-5 and Siglec-9 (Figure 6.5B). Both Siglec-5 and Siglec-9 are thought to possess immunoinhibitory functions (386). Furthermore, their activation has recently been linked to IL-10 expression in murine macrophages (397). We also detected phosphorylation of SH2 domain-containing tyrosine phosphatase (SHP-2), a signalling factor recruited by the immunoreceptor tyrosine-based inhibitory motif (ITIM) cytoplasmic domains of Siglec receptors, which mediates immunosuppressive signalling (251). Two other immunoinhibitory receptors became phosphorylated in the presence of WT *C. jejuni*; platelet endothelial cell adhesion molecule (PECAM) (also known as CD31), which is known to inhibit pro-inflammatory signalling though recruitment of SHP-2 (398), and

Figure 6.5. *C. jejuni* induces phosphorylation of Siglec-5 and Siglec-9.
dTHP-1 cells were co-cultured with WT *C. jejuni* 1168H MOI 100 for 60min. (A) Receptor activation was analysed using an Immunoreceptor Phosphorylation Array kit by R&D. Image shown is representative of three independent experiments. (B) Pixel density analysis of data (60min) shown as values ± SEM. Pixel density values between images were normalized using the reference point readings.

FcγRIIA, a molecule that can also recruit SHP-2 via its ITIM domain, leading to inhibition of phagocytosis (399). Since only low levels of phospho-SHP-2 were detected, induction of SHP-2 phosphorylation in response to *C. jejuni* infection was confirmed by flow-cytometry (Figure 6.6). Following 1h co-culture of dTHP-1 cells with *C. jejuni,* increased expression of phosphorylated SHP-2 was observed Our data suggests that *C. jejuni* infection activates Siglec-5 and Siglec-9, as well as the inhibitory receptors PECAM and FcγRIIA, followed by recruitment of SHP-2 to mediate downstream immunosuppressive functions.

6.2.3 Siglec-5 receptor engagement by *C. jejuni*

Naturally, we wanted to decipher the physical interaction that led to the strong activation of Siglec-5 and Siglec-9 by *C. jejuni*. An immunoabsorbance-based technique similar to traditional ELISA was used to analyse binding affinity between the bacteria and recombinant Siglec proteins. To study SA, a low affinity ligand for Siglecs, involvement in Siglec binding the SA-negative *NeuB C. jejuni* was used. in addition, bacteria were treated with sialidase from *Arthrobacter ureafaciens* which hydrolyses SA α2,3-, α,2,6- or α,2,8-linked to Neu5Ac. The capsule-negative *kpsm* mutant of *C. jejuni* was also used based on the hypothesis that the lack of a capsule would further expose SA ligands. Increased binding of Siglec 5 was only observed in the capsule deficient mutant compared to all others tested (P=0.032), Moreover, an absence of SA did not affect Siglec-5 binding to the pathogen as similar OD readings were observed for the SA-negative and WT stain (Figure 6.7). This was further confirmed by no difference following sialidase treatment suggesting that this interaction is SA independent. Therefore, Siglec-5 appears to recognise *C. jejuni* motifs concealed by the bacterial capsule.

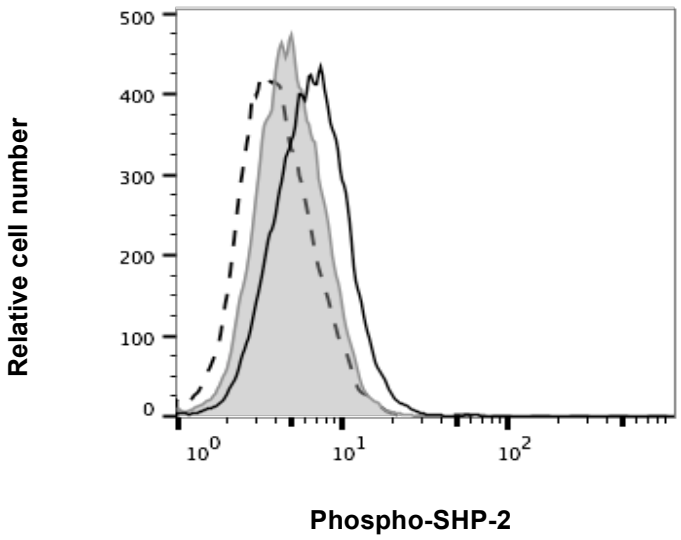

Figure 6.6. *C. jejuni* induces phosphorylation of SHP-2 kinase.

dTHP-1 cells were co-cultured with WT *C. jejuni* 1168H MOI 100 for 60min. SHP-2 phosphorylation in uninfected (grey solid line) and infected (black line) was analysed by flow cytometry. IgG isotype staining (dashed line) was used as control. Representative histogram from three independent experiments is shown.

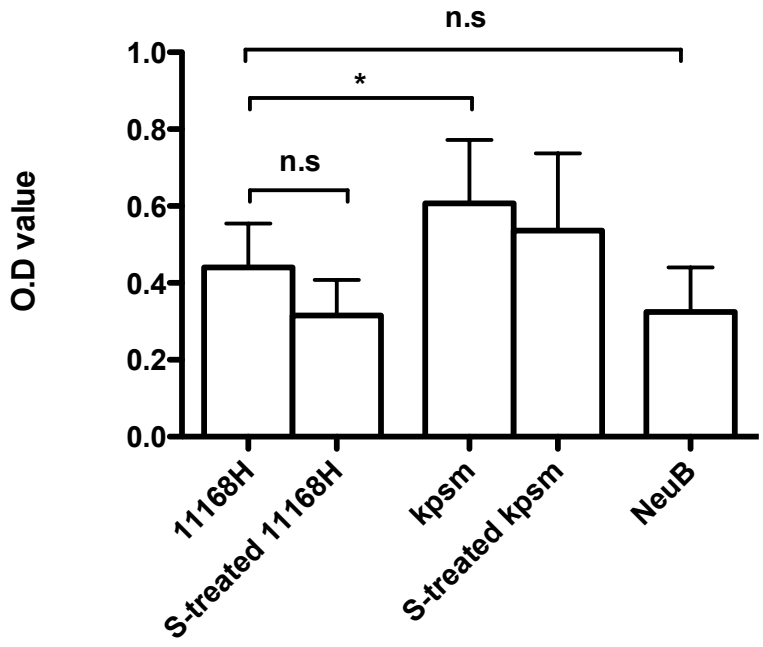

Figure 6.7. Siglec-5 recognises motifs concealed by the capsule of *C. jejuni*.
Untreated or sialidase-treated (S-treated) WT or *kpsm*, or *NeuB C. jejuni* strain 11168H were coated onto the wells of ELISA plates and direct binding of hSiglec-5-Fc was assayed by incubation with 2µg/ml recombinant protein. Bound protein was detected using avidin-HRP and analysed in a photometric reader (450nM). Values are means ± SEM from at least three independent experiments in duplicate (*P=0.032).

To explore these interactions further, we over-expressed the Siglec-5 and Siglec-9 receptors on the human kidney CHO cell line. Transduction at MOI 100 led to ~50% of the cells being positive for Siglec-5 (Figure 6.8). Siglec-9 transduction efficiency was comparable to Siglec-5 at ~48% (Figure 6.9). Siglec-10 CHO cells were used as a positive control since they have been shown to specifically bind *C. jejuni* (218). When co-cultured with FITC-labelled bacteria, Siglec-5 CHO cells had more *C. jejuni* adhere to them, with ~23% FITC positive compared to only ~14% FITC positive of WT CHO cells (Figure 6.10). However, it is worth noting that the Siglec-10 CHO cell population is uniform as opposed to the Siglec-5 population were only half of them were positive for Siglec-5. Therefore we would expect a higher number of FITC-positive cells in both cell lines had uniform populations been used. Unfortunately, the plasmids used in transductions did not contain any "tag" proteins (e.g GFP) and we were therefore unable to sort these cells into pure populations. The specificity of *C. jejuni* binding to Siglec-5 CHO cells was confirmed using a neutralising/blocking (N/B) antibody against the receptor. The cells were treated with anti-Siglec-5 N/B antibody prior to co-culture with FITC-labelled *C. jejuni* carried out at 4^{o}C to avoid endocytosis of the receptors. Blocking of Siglec-5 led to a decrease in Siglec-5-mediated binding of *C. jejuni* from ~23% to ~10% of cells, but had no effect on the binding to Siglec-9 CHO cells (Figure 6.11). These data further support the specificity of *C. jejuni* binding to Siglec-5.

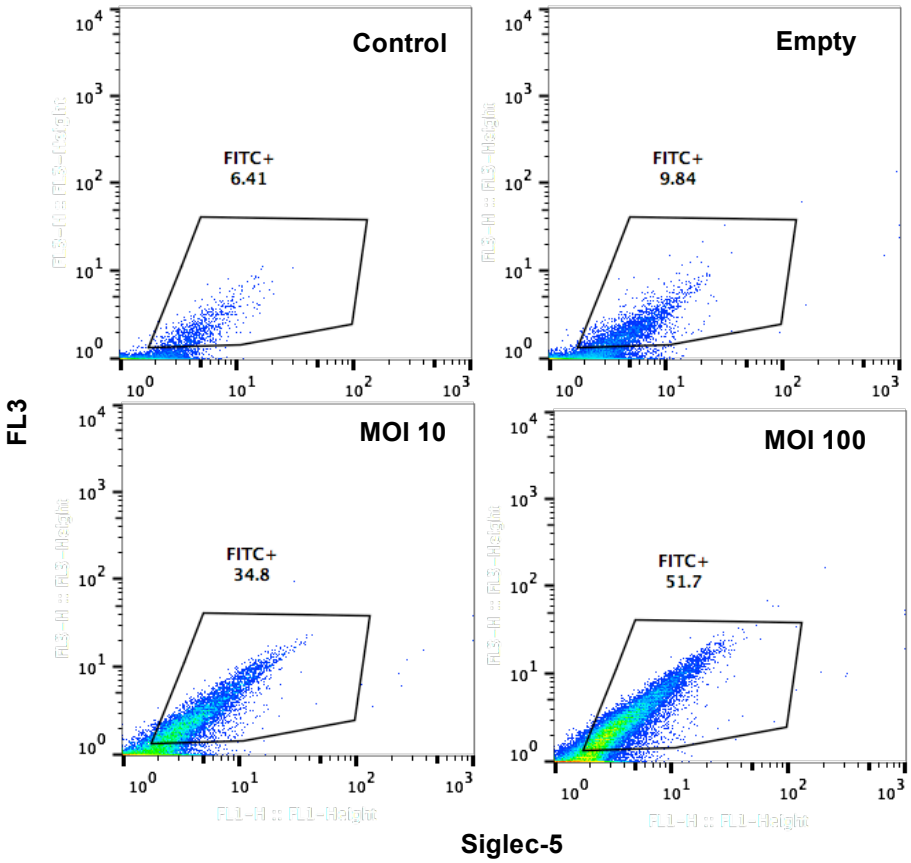

Figure 6.8. Siglec-5 receptor overexpression in CHO cells.

CHO cells were transduced with empty and Siglec-5 (MOI 10 and 100) vectors. After 72h, cells were stained with anti-hSiglec-5 antibody conjugated to FITC anti-human IgG (which isotype?) antibody and analysed by flow cytometry. Representative histogram of one experiment performed in duplicate is shown.

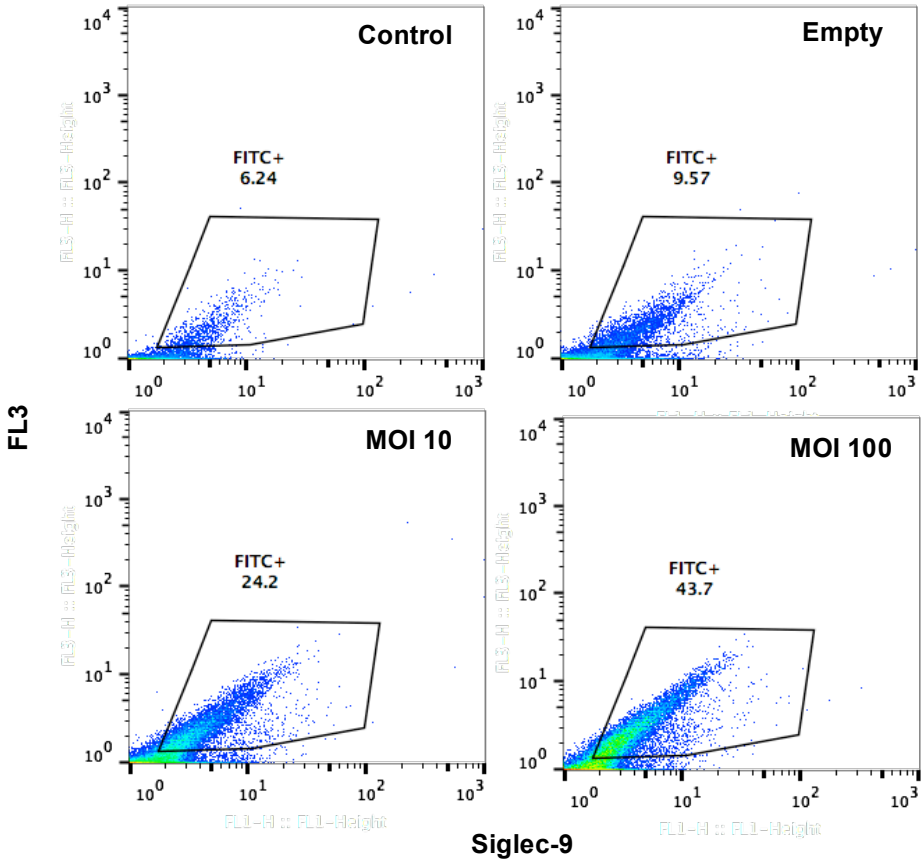

Figure 6.9. Siglec-9 receptor overexpression in CHO cells.

CHO cells were transduced with empty and Siglec-9 (MOI 10 and 100) vectors. After 72h, cells were stained with anti-hSiglec-9 antibody conjugated to FITC anti-human IgG antibody and analysed by flow cytometry. Representative histogram of one experiment performed in duplicate is shown.

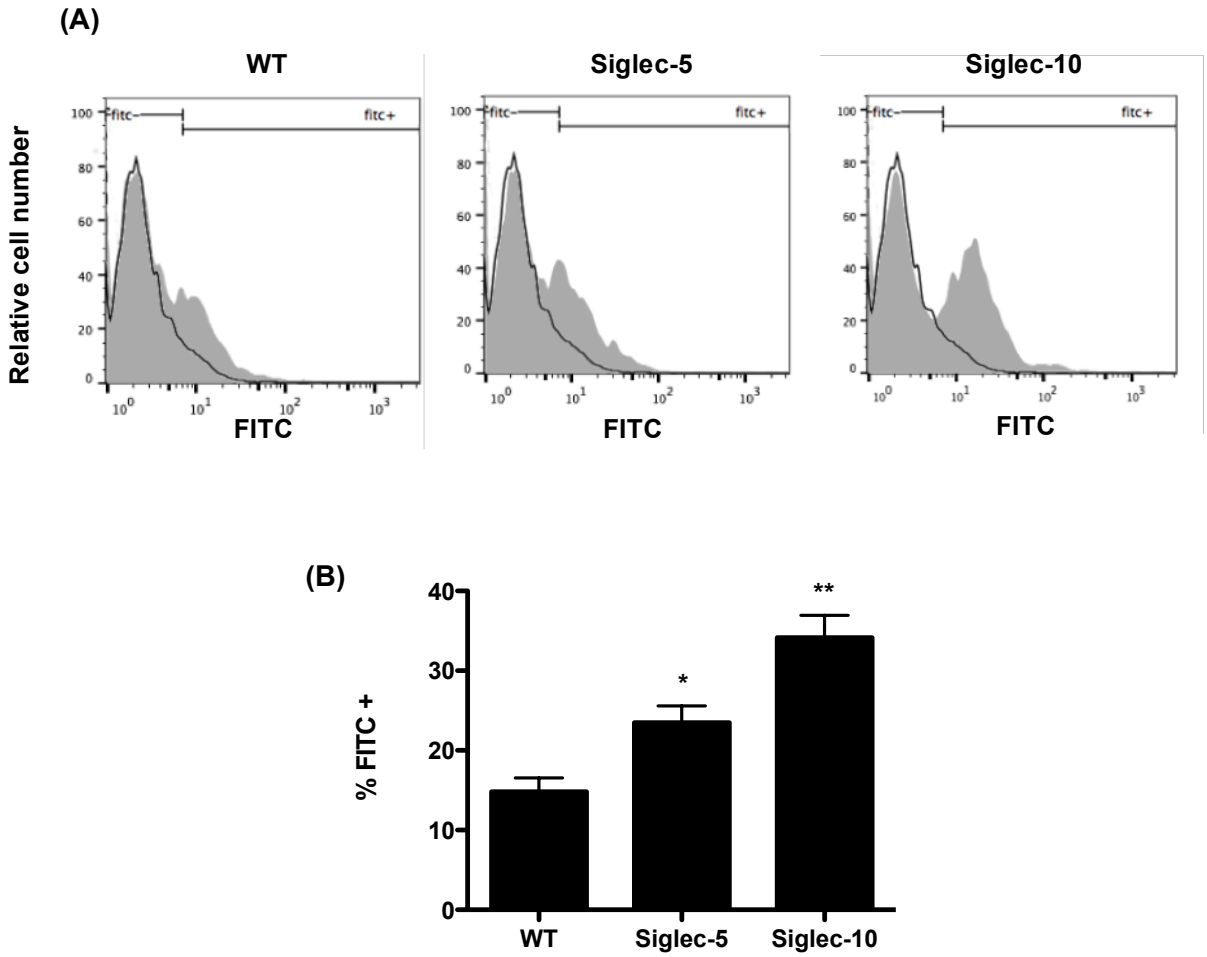

Figure 6.10. *C. jejuni* binds specifically to Siglec-5-overexpressing CHO cells. WT and Siglec-5-overexpressing CHO cells were mixed with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C to prevent internalisation. Siglec-10 CHO cells were used as a control for *C. jejuni* binding. (A) After 2h, FITC-positive cells were analysed by flow cytometry (representative histogram shown). (B) Data shown as percentage of FITC-positive cells. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

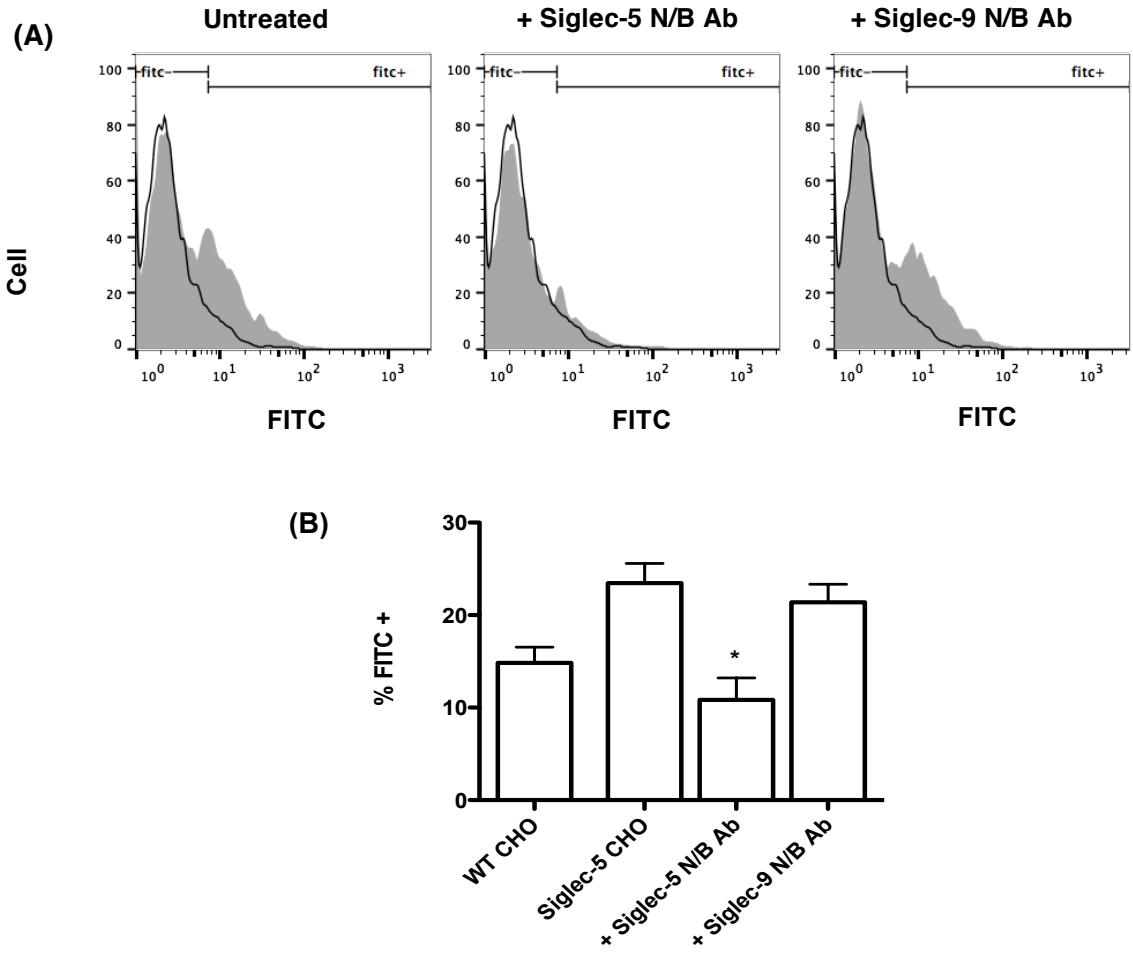

Figure 6.11. Siglec-5 blocking antibody specifically inhibits *C. jejuni* binding to the receptor.

Siglec-5-overexpressing CHO cells were pre-treated with anti-hSiglec-5 blocking antibody for 1h prior to mixing with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C. Anti-hSiglec-9 blocking antibody served as non-specific control. (A) After 2h, FITC-positive cells were analysed by flow cytometry (representative histograms shown). (B) Data shown as percentage of FITC-positive cells. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test ($P<0.05$).

Next, the Siglec-5 CHO cell lines were used to confirm our earlier evidence of structure-specific recognition of *C. jejuni* by the two receptors. Again, the capsule negative *kpsm* and SA negative *NeuB* mutants of *C. jejuni* were utilised to test for capsule and SA-dependent interactions respectively. We observed significantly increased binding of the *kpsm* mutant to Siglec-5 CHO cells (P=0.03), while adherence of *NeuB C. jejuni* was not significantly different to the WT strain (Figure 6.12). This finding reinforces previous evidence of capsule-independent binding of *C. jejuni* to Siglec-5 (see Figure 6.7).

Next, we wanted to delineate the functional aspects of Siglec-5 engagement by *C. jejuni*. Studies have shown that engagement of Siglecs by a pathogen can lead to suppression of phagocytosis in human APCs (264),(265). We used dTHP-1 cells to investigate the effects of *C. jejuni*-mediated Siglec engagement on bacterial adherence to and internalisation by human macrophages. The cells were pre-treated with N/B antibody for Siglec-5 prior to co-culture with *C. jejuni* strain 11168. Blocking of Siglec-5 led to a significant decrease in bacterial binding (P=0.0249) (Figure 6.13A), indicating the importance of Siglec-5 for the adherence of *C. jejuni* to dTHP-1 cells; similar to the results of our binding assay (see Figure 6.12). Blocking of Siglec-5 also led to a significant reduction in *kpsm C. jejuni* binding (P=0.0309) (Figure 6.13B); suggests the interaction is independent of the capsule. Finally, the *kpsm* mutant exhibited increased binding to dTHP-1 cells (~75% FITC-positive cells) compared to WT *C. jejuni* (~67% FITC-positive cells), reflecting the increased affinity of Siglecs for *kpsm C. jejuni* demonstrated in our ELISA experiment (Figure 6.7).

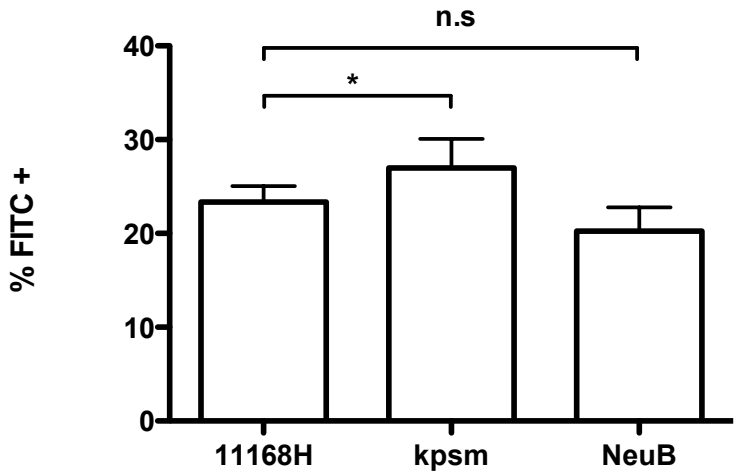

Figure 6.12. *C. jejuni* binding to Siglec-5 is capsule-independent.

Siglec-5-overexpressing CHO cells were mixed with FITC-labelled WT, *kpsm* or *NeuB C. jejuni* strain 11168H MOI 100 at 4°C. After 2h, FITC-positive cells were analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test ($P<0.05$).

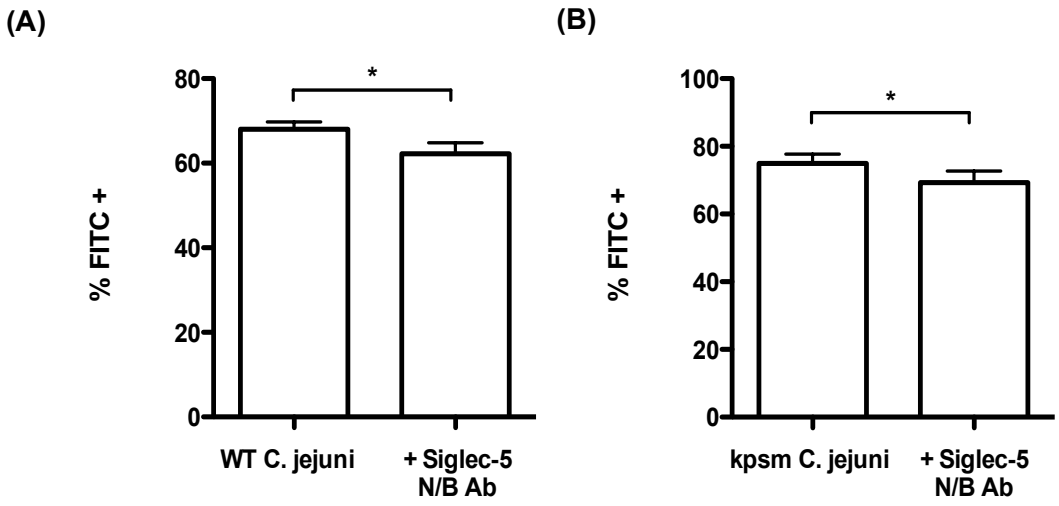

Figure 6.13. Siglec-5 is important for capsule-independent *C. jejuni* adherence to dTHP-1 cells.

dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-5 N/B antibody for 1h prior to co-culture with FITC-labelled (A) WT or (B) *kpsm C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, FITC-positive cells were analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

With the primary function of a number of Siglecs being the recognition of 'self' (253), we hypothesised that engagement of Siglec-5 by the cell's own sialylated ligands may disrupt the engagement of the receptor to *C. jejuni*. Therefore, dTHP-1 cells were treated with sialidase from *Clostridium perfingens* which preferentially cleaves α2,3-linked SAs from Neu5Ac in order to disengage glycan receptors bound to SA. Subsequently, cells were incubated with N/B antibody against Siglec-5 prior to co-culture with WT *C. jejuni* strain 11168. As Figure 6.14 shows, blocking of Siglec-5 led to a more significant reduction in *C. jejuni* adherence to sialidase-treated (P=0.008) compared to untreated (P=0.025) dTHP-1 cells.

To further explore the dynamics of this interaction, *C. jejuni* and dTHP-1 cells were co-cultured at room temperature where the phagocytic as well as signalling processes are active. Here, blocking of Siglec-5 led to a statistically significant reduction in the total number of bacteria associated with dTHP-1 cells (P=0.003) (Figure 6.15A). A similar reduction was observed using *kpsm C. jejuni* (P=0.006), mirroring our earlier observation of a capsule-independent interaction between bacteria and cells (see Figure 6.13). On the other hand, numbers of intracellular bacteria, for both WT and *kpsm* mutant *C. jejuni,* were unaffected by blocking of the receptor (Figure 6.15B), although a small, but not significant, reduction was recorded. Therefore, interaction with Siglec-5 appears to be more important in *C. jejuni* adherence to rather than internalisation by macrophages.

6.2.4 Siglec-9 receptor engagement by *C. jejuni*

A similar experimental approach to the one detailed above (see Paragraph 6.4) was used to identify potential interactions between *C. jejuni* and Siglec-9. Employing the

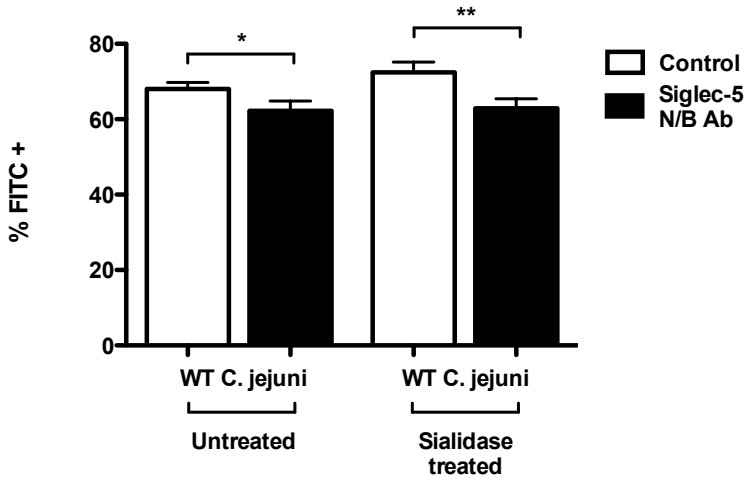

Figure 6.14. Sialidase treatment affects Siglec-5-mediated *C. jejuni* adherence in dTHP-1 cells.

Untreated or sialidase-treated dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-5 N/B for 1h prior to co-culture with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, the presence of bacteria on cells was analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

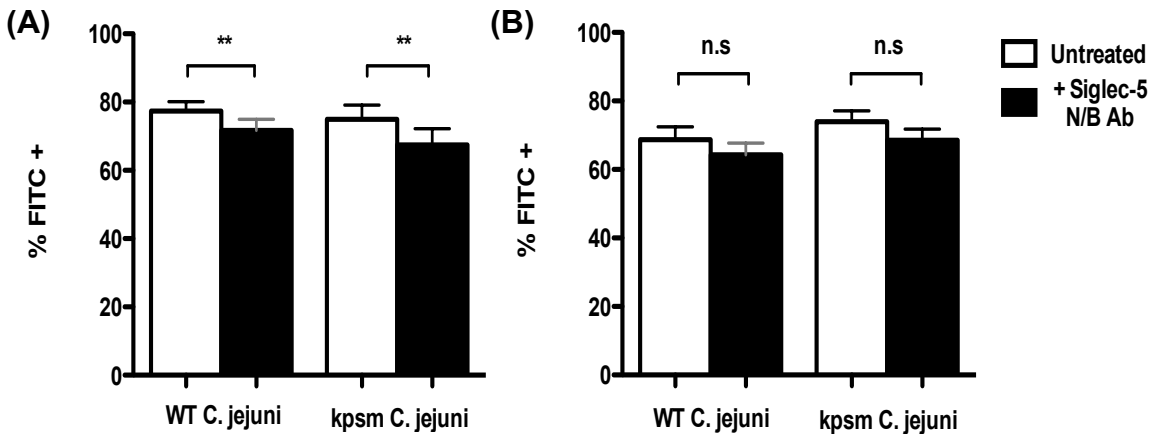

Figure 6.15. Engagement of Siglec-5 alters the interaction of *C. jejuni* with dTHP-1 cells.

dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-5 N/B antibody for 1h prior to co-culture with FITC-labelled WT or *kpsm C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, (A) total and (B) intracellular bacterial presence was analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (**P<0.01).

ELISA assay discussed in Figure 6.7, we were able to demonstrate binding of recombinant Siglec-9 to *C. jejuni* strain 11168. Binding was SA-dependent as there was a statistically significant reduction in Siglec-9 binding in the absence of SA; either following sialidase treatment (P=0.012) or against the *NeuB* mutant (P=0.011) (Figure 6.16). Similar to Siglec-5, lack of a capsule led to a statistically significant increase in Siglec-9 binding (P=0.044), although this interaction was SA-mediated as it was sialidase-sensitive. Therefore, Siglec-9 appears to recognise SA structures on the pathogen.

In binding assays using transduced CHO cells, Siglec-9 CHO cells had more *C. jejuni* adhere to them, with ~36% FITC-positive cells compared to only ~14% of WT CHO cells (Figure 6.17); a nearly three-fold increase. Although binding appears to be equal between Siglec-9 and Siglec-10 CHO cells, with ~36% and ~34% FITC positive respectively, only the Siglec-10 CHO cell population is uniform as discussed earlier. The specificity of *C. jejuni* binding to Siglec-9 overexpressing CHO cells was confirmed using blocking antibodies against the receptors. Blocking of Siglec-9 reduced *C. jejuni* binding to Siglec-9 CHO cells, from ~36% to ~14% without altering adherence to Siglec-5 CHO cells (Figure 6.18). In Siglec-9 CHO cells, lack of SA led to a significant reduction in bacterial binding (P=0.0148) (Figure 6.19); confirming that the interaction between the receptor and *C. jejuni* is SA-dependent. Binding was unaffected by the lack of a capsule, although we would expect an increase based on our ELISA data (see Figure 6.16). A likely explanation for this discrepancy is that bacterial binding becomes saturated by WT *C. jejuni* and therefore our analysis does not reflect increased affinity for the *kpsm* mutant. We confirmed that SA is the *C. jejuni* ligand recognised by Siglec-9 though a competitive binding assay using

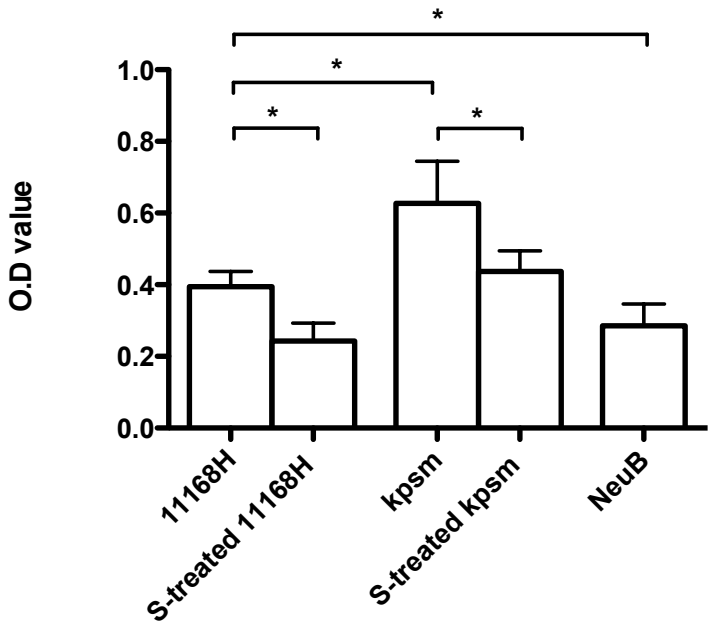

Figure 6.16. Siglec-9 recognises sialic acid structures on *C. jejuni*.

Untreated or sialidase-treated (S-treated) WT or *kpsm*, or *NeuB C. jejuni* strain 11168H were coated onto the wells of ELISA plates and direct binding of hSiglec-9-Fc was assayed by incubation with 2µg/ml recombinant protein. Bound protein was detected using avidin-HRP and analysed in a photometric reader (450nM). Values are means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis by paired t-test (*$P<0.05$).

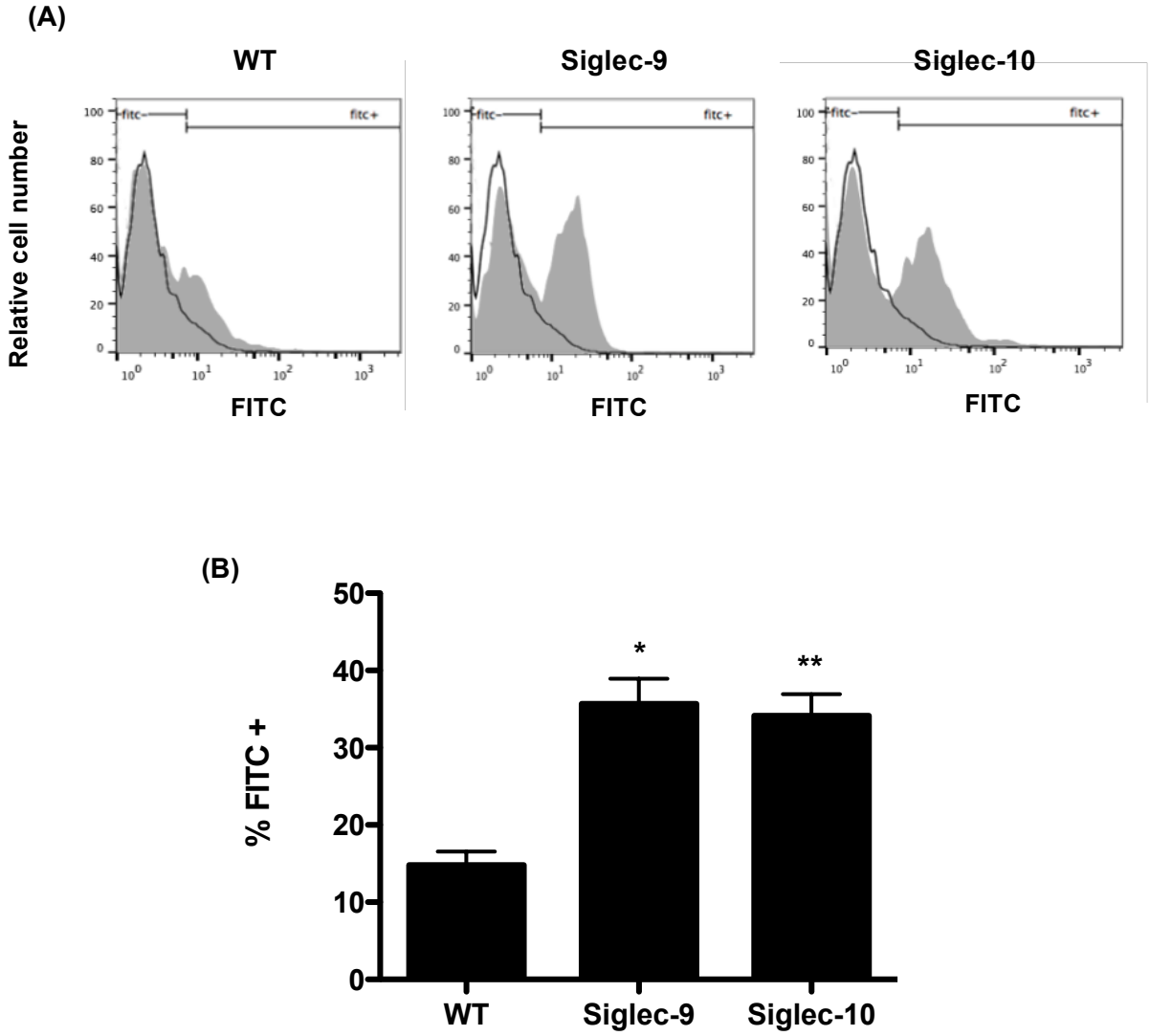

Figure 6.17. *C. jejuni* binds specifically to Siglec-9-overexpressing CHO cells.

WT and Siglec-9-overexpressing CHO cells were mixed with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C to prevent internalisation. Siglec-10 CHO cells were used as a control for *C. jejuni* binding. (A) After 2h, FITC-positive cells were analysed by flow cytometry (representative histogram shown). (B) Data shown as percentage of FITC-positive cells. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

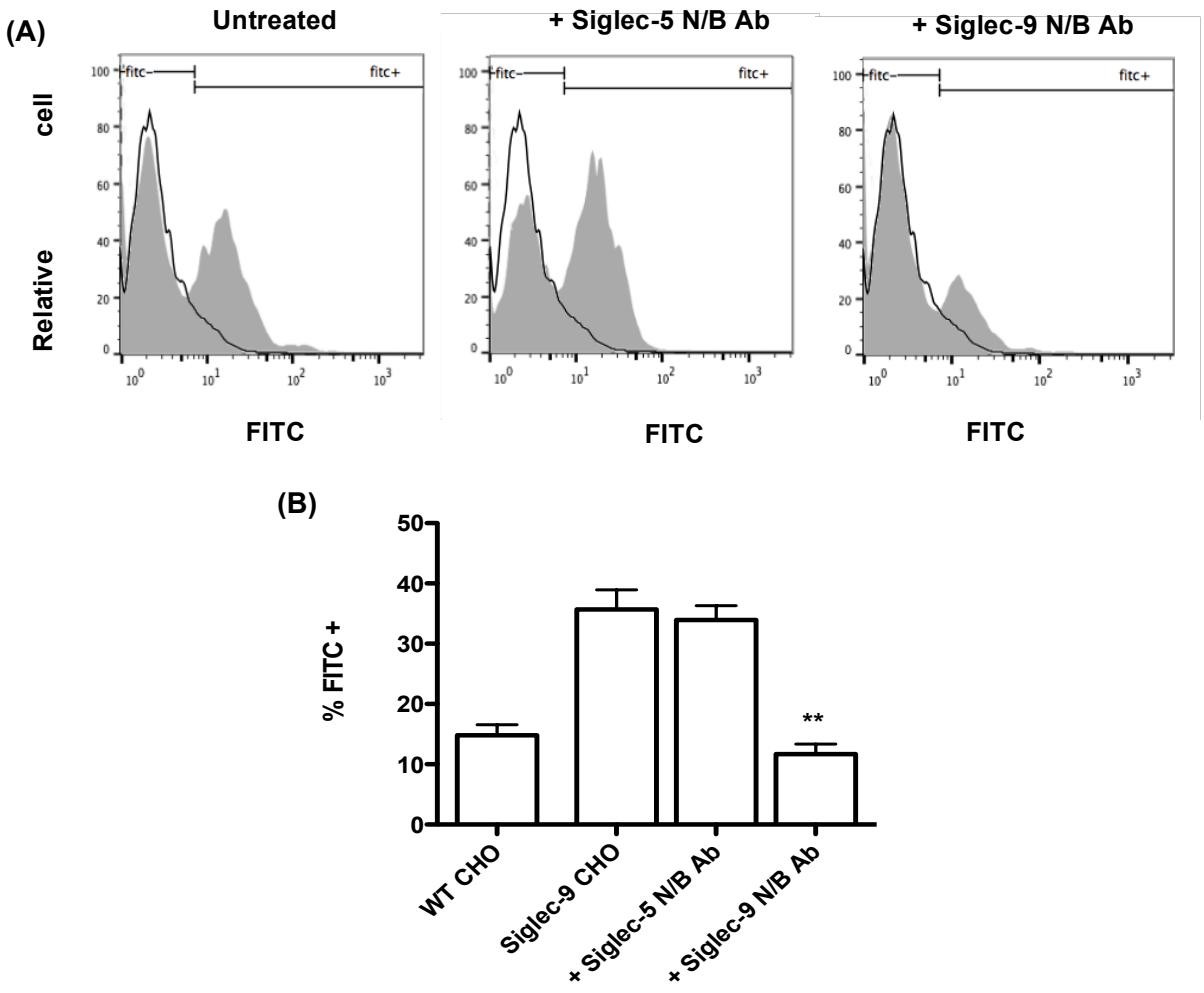

Figure 6.18. Siglec-9 blocking antibody specifically inhibits *C. jejuni* binding to the receptor.

Siglec-9-overexpressing CHO cells were pre-treated with anti-hSiglec-9 blocking antibody for 1h prior to mixing with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C. Anti-hSiglec-5 blocking antibody served as non-specific control. (A) After 2h, FITC-positive cells were analysed by flow cytometry (representative histograms shown). (B) Data shown as percentage of FITC-positive cells. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (**P<0.01).

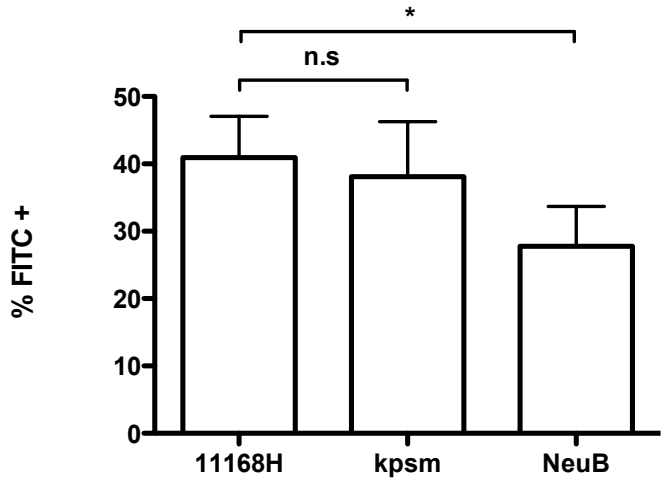

Figure 6.19. *C. jejuni* binding to Siglec-9 is sialic-acid-dependent.

Siglec-9-overexpressing CHO cells were mixed with FITC-labelled WT, *kpsm* or *NeuB C. jejuni* strain 11168H MOI 100 at 4°C. After 2h, FITC-positive cells were analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

isolated LOS of *C. jejuni*. Since *C. jejuni* 11168 LOS is monosialylated it should ligate to Siglec-9 and compete with *C. jejuni* for binding to CHO cells. Indeed, pre-treatment of Siglec-9 CHO cells with 0.1µg/ml LOS led to a significant reduction in *C. jejuni* binding (P=0.0148), nearly 50% less compared to untreated cells (Figure 6.20). We therefore conclude that Siglec-9 binds to the SA structures on *C. jejuni* LOS.

Whereas Siglec-9 was involved in *C. jejuni* adherence to CHO cells (Figure 6.19), it played no role in binding to macrophages, since pre-treatment of dTHP-1 cells with N/B antibody for Siglec-9 prior to co-culture with WT or *kpsm C. jejuni* strain 11168 had no significant effect (Figure 6.21). Nevertheless, in sialidase-treated cells, Siglec-9 appears to play a part in the pathogen's interaction with dTHP-1 cells, as a significant reduction in bacterial binding (P=0.02) was observed following Siglec-9 blocking (Figure 6.22). Overall, in contrast to untreated dTHP-1 cells, both Siglec receptors were important for *C. jejuni* adherence to sialidase treated cells. When the experiment was carried out at room temperature, Siglec-9 blocking led to a significant reduction in the number of total bacteria, either WT (P=0.031) or *kpsm* (0.012) *C. jejuni*, associated with dTHP-1 cells (Figure 6.23). Similar to the blocking of Siglec-5, there was not effect on the number of internalised bacteria. This may come as a surprise given that Siglec-5 and Siglec-9 act to inhibit phagocytosis and we could, therefore, expect an increase in the number of internalised bacteria after blocking these receptors. However, at this stage, it is not known how these blocking antibodies may affect the functions of these receptors.

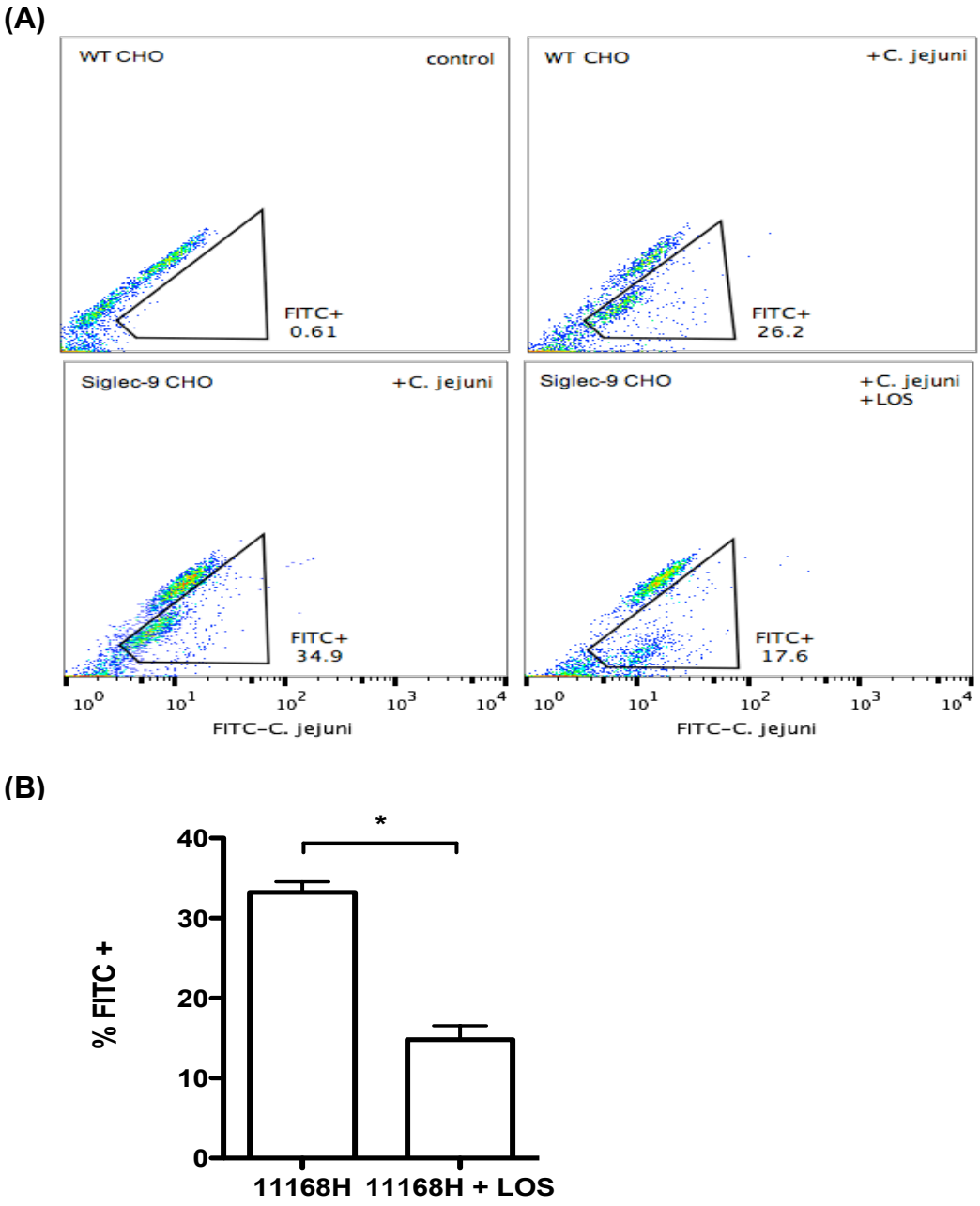

Figure 6.20. *C. jejuni* binding to Siglec-9 is LOS-dependent.

Siglec-9-overexpressing CHO cells were left untreated or treated with 100ng/ml LOS from *C. jejuni* strain 11168H at 4°C to prevent ligand internalisation. After 1h, cells were mixed with FITC-labelled *C. jejuni* strain 11168H MOI 100 at 4°C for 1h and FITC-positive cells analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

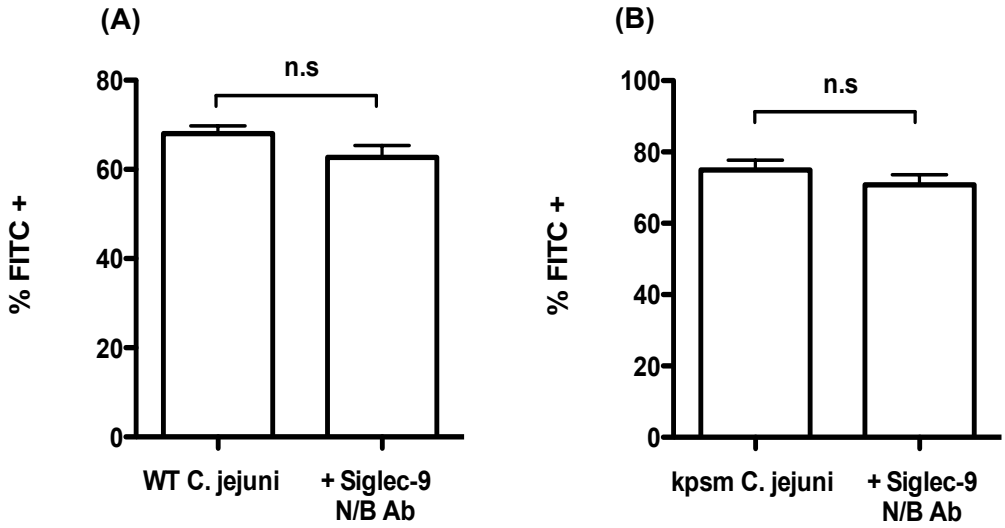

Figure 6.21. Siglec-9 is not involved in *C. jejuni* adherence to dTHP-1 cells.

dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-5 N/B or anti-hSiglec-9 N/B antibody for 1h prior to co-culture with FITC-labelled (A) WT or (B) *kpsm C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, FITC-positive cells were analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

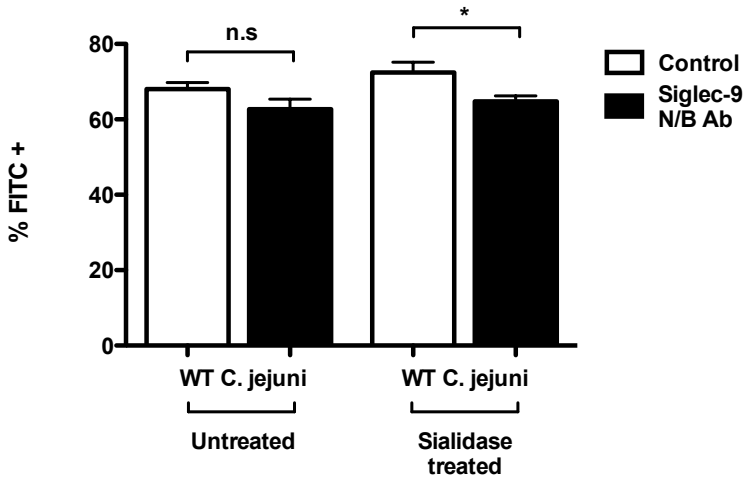

Figure 6.22. Sialidase treatment affects Siglec-9-mediated *C. jejuni* adherence in dTHP-1 cells.

Untreated or sialidase-treated dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-5 N/B for 1h prior to co-culture with FITC-labelled WT *C. jejuni* strain 11168H MOI 100 at 4°C to prevent bacteria internalisation. After 1h, the presence of bacteria on cells was analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05, **P<0.01).

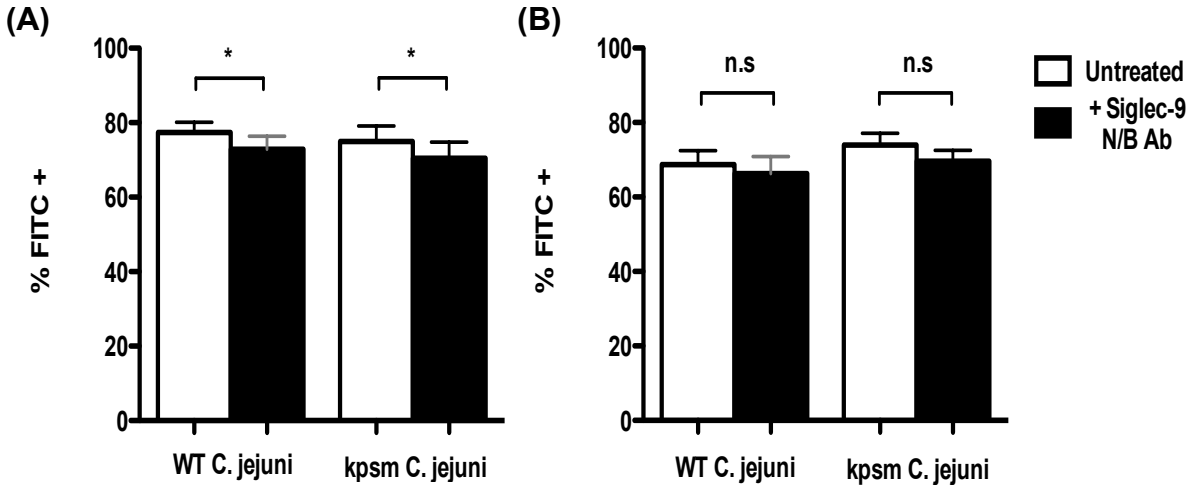

Figure 6.23. Engagement of Siglec-9 alters the interaction of *C. jejuni* with dTHP-1 cells.

dTHP-1 cells were pre-incubated with 5µg/ml anti-hSiglec-9 N/B antibody for 1h prior to co-culture with FITC-labelled WT or *kpsm C. jejuni* strain 11168H MOI 100. After 1h, (A) total and (B) intracellular bacterial presence was analysed by flow cytometry. Values are means ± SEM from three independent experiments performed in duplicate. Statistical analysis by paired t-test (*P<0.05).

6.6 Discussion

Although we initially set out to characterise the role of Siglec-10 in the cytokine response to *C. jejuni* strain 11168, our study revealed the involvement of multiple other Siglec receptors in sensing the pathogen. *C. jejuni* strain 11168 triggered the phosphorylation of Siglec-3, Siglec-5 and Siglec-9 in dTHP-1 macrophage-like cells (Figure 6.5). Crocker and colleagues first demonstrated engagement of Siglec receptors by *C. jejuni* - predominantly Siglec-7 - although weak binding by Siglec-5, Siglec-9 and Siglec-10 was also detected (262). Siglecs are considered as immunomodulatory receptors thought to be important in distinguishing "danger-associated" versus "pathogen-associated" signals (261). The capacity of pathogens to sialylate their surface and engage Siglecs offers them an evolutionary advantage by dampening the immune response. This is the first report of Siglec-3 activation by *C. jejuni*, although that was weak compared to Siglec-5 and Siglec-9 and was not investigated further. *Group B streptococcus* (GBS) and *Pseudomonas aeruginosa* are known to engage Siglec-9 on neutrophils and reduce their oxidative burst and extracellular trap formation (265),(400). GBS also binds to Siglec-5 leading to suppression of phagocytosis in human leukocytes and neutrophils (264). Furthermore, both receptors have been linked to the production of the anti-inflammatory cytokine IL-10. Siglec-9 and Siglec-5 overexpression in the macrophage cell-lines RAW264 and THP-1, followed by TLR stimulation led to an increase in IL-10 production with a parallel decrease in TNFα (401). Siglec-9-mediated stimulation of IL-10 is mediated by tyrosine-based motifs (401) and is partly due to an increase in intracellular C/EPβ levels (397). Activation of Siglec-5 and Siglec-9 may account for the strong induction of IL-10 by *C. jejuni* compared to other enteropathogens (218).

The other ITIM-containing Siglec receptors did not become phosphorylated. Previous studies have demonstrated Siglec-7 preference for disialylated a2,3/2,8 motifs (262),(393), therefore we did not expect *C. jejuni* 11168 to activate the receptor. Surprisingly, neither Siglec-10 or its associated SHP-1 phosphatase (402) were phosphorylated despite evidence of *C. jejuni* binding to Siglec-10 (262),(218). However, Siglec-10 ligation may lead to activation of alternative domains as discussed below.

GBS engagement of ITIM-bearing Siglec-5 or Siglec-9 activates inhibitory SHP2-dependent signals that interfere with cellular activation. In cells co-cultured with *C. jejuni* we detected phosphorylation of SHP-2 but not SHP-1 (Figure 6.5 and Figure 6.6). SHP-2 has a dual role in immune cell regulation. While SHP-2 recruited by ITIM-bearing receptors can inhibit immune cell activation (403),(404), it is also a positive regulator of cytokine and growth factor receptor signalling, mediating Ras/MAPK and ERK activation (405). ITIMs differ in their affinity for SHP-1 and SHP-2, and specific recruitment may contribute to inhibitory capacity. *Helicobacter pylori*, a pathogen closely related to *C. jejuni*, also activates SHP-2 by injecting cytotoxin-associated antigen A (cagA) protein into host cells to suppress INF-γ signalling (406),(407). Others have demonstrated the capacity of *H. pylori* to induce crosstalk between TLRs and the C-type lectin DC-SIGN that leads to high levels of IL-10 production by DCs (270). Intriguingly, SHP-2 is an oncoprotein and CagA-mediated deregulation of SHP-2 is associated with severe gastritis and gastric carcinoma (408). It is compelling to hypothesise that activation of immunosuppressive mediators such as SHP-2 by certain pathogens can lead to a persistent imbalance in host cell signalling that may lead to pathogenesis.

Two more immunosuppressive factors became phosphorylated; PECAM-1 and FcγRIIA. PECAM-1 is a negative regulator of TLR4 signalling, suppressing the TNFα and IL-6 response to LPS (409). Phosphorylated FcγRIIA is able to recruit SHP-1 and SHP-2 (399) and, like Siglec-5, can inhibit Fc receptor-mediated phagocytosis (410). This is the first report of *C. jejuni*-mediated activation of these factors and further work is required to decipher their contribution in the macrophage response to the pathogen.

We went on to identify the *C. jejuni* structures involved in Siglec-5 and Siglec-9 engagement. By over-expressing each receptor (Figure 6.8 and Figure 6.9), we detected increased binding of WT *C. jejuni* to Siglec-5 (Figure 6.10) and Siglec-9 (Figure 6.17) over-expressing CHO cells. Binding to Siglec-9 was more prominent compared to Siglec-5, similar to findings in other *C. jejuni* strains (262), although we aim to repeat these studies in uniform cell populations in the future. Critically, using neutralising/blocking antibodies to block the receptor's binding site we were able to demonstrate Siglec-5 and Siglec-9-specific binding to *C. jejuni* 11168 (Figure 6.10 and Figure 6.18).

Studies on GBS have reported Siglec receptor binding to sialylated capsular and LOS structures on pathogens (258),(265). We found increased interaction of Siglec-5-Fc chimera to the acapsular *kpsm* mutant compared to WT *C. jejuni* (Figure 6.7). Importantly, the binding affinity was unaffected by the removal of SA or use of the SA-negative *NeuB* mutant, indicating that Siglec-5 interaction with *C. jejuni* is independent of SA moieties. Accordingly, we observed increased binding of *kpsm C. jejuni* to Siglec-5 (Figure 6.12) but not Siglec-9 overexpressing cells (Figure 6.19), leading us to hypothesise that Siglec-5 ligands are concealed by the bacterial capsule. Recent studies suggest that in addition to SA, Siglecs can also ligate to non-sialylated ligands. For example, Siglec-5 recognizes Group B *Streptococcus* β-protein (265), and Siglec-

10 can interact with vascular adhesion protein-1 (259). It would be interesting to identify the Siglec-5 ligand(s) by utilising various *C. jejuni* mutants in similar binding assays.

On the other hand, *C. jejuni* engagement to Silgec-9 was SA-dependent, as lack of SA led to a reduction in Siglec-9-Fc chimera binding (Figure 6.16). Furthermore, we observed decreased binding of the SA-negative *NeuB C. jejuni* to Siglec-9 over-expressing CHO cells compared to the WT strain (Figure 6.19), indicating that sialylated LOS may act as a ligand for the receptor. This was confirmed by showing that *C. jejuni* LOS competes with the pathogen for Siglec-9 binding (Figure 6.20). This first report of Siglec-9 binding to GM1 or GM2-like epitopes is intriguing and may help explain why although Siglec-9 exhibits binding preference for GD1a-like motifs (411), it cannot recognise GD1a LOS (262). Sn is the only other Siglec reported to bind GM1α-like structures on *C. jejuni* (263),(394). Siglec-9 binds to the capsular polysaccharide of GBS which also presents terminal sialylated α2,3-Galβ1-4GlcNAc units (265). The weak affinity for *C. jejuni* can likely be attributed to the presence of less accessible "internal", rather than terminal, SA residues in GM1- and GM2-like epitopes (394). Using purified *C. jejuni* LOS treated with sialidase enzymes of different affinities in competitive binding assays as the above (see Figure 6.20) should reveal the SA recognised by Siglec-9. Similar to Siglec-5, we observed an increase in Siglec-9 binding to *kpsm C. jejuni* (Figure 6.16); likely a result of sialylated ligands located beneath the capsule. Others have reported on the negative effect of the capsule on the interaction of *C. jejuni* with host cell receptors (412) and in our hands it interfered with bacterial ligation to both Siglec-5 and Siglec-9.

We also looked at the role of these two receptors in the interaction of *C. jejuni* with a model of human macrophages. Although expression of Siglecs has not been reported in macrophages (256), high levels of phosphorylated Siglec-5 and Siglec-9 were

detected in dTHP-1 cells(Figure 6.5). Siglec receptor expression in this cell line requires further study. Alternatively, neutrophils may offer a more relevant model for the study of *C. jejuni* – Siglec interactions as they are known to express both receptors (413),(414). Furthermore, bacteria such as Group B *Streptococcus*, *E. coli* and *P. aeruginosa* and are thought to interact with Siglec-5 and Siglec-9 on neutrophils to inhibit phagocytosis (415),(400),(416).

Surprisingly, only Siglec-5 ligation appeared to be important for *C. jejuni* adherence to dTHP-1 cells (Figure 6.13 and Figure 6.21), despite the affinity exhibited by Siglec-9 in our ELISA experiment (Figure 6.16). Studies have demonstrated that Siglec receptors engage in cis interactions with endogenous ligands and as a result binding may be blocked or "masked" (134),(417). Treatment with sialidase led to an increase in both Siglec-5 and Siglec-9-mediated adherence of *C. jejuni* (Figure 6.14 and Figure 6.22) and therefore, we consider both to be important in the pathogen's interaction with macrophages. It would be worth undertaking these experiments again using different blocking antibodies or even silencing of Siglec genes by RNA interference.

Bacterial sialylation is thought to affect phagocytosis of microorganisms, such as GBS (264) and *C. jejuni*; with sialylated *C. jejuni* being phagocytosed more efficiently in vitro by bone-marrow macrophages (389). Furthermore, CD33-related Siglecs are thought to be involved in the uptake and processing of soluble and particulate ligands (418) and in Siglec-9, the endocytotic function was shown to be ITIM-dependent (419). Whereas the number of total bacteria present in dTHP-1 cells was reduced, we did not detect a difference in the number of intracellular pathogens (Figure 6.15 and Figure 6.23). This was somewhat unexpected since others have reported Siglec-5- and Siglec-9-mediated suppression of phagocytosis in leukocytes infected with GBS (264),(415). However, it may be worth noting that the immunosuppressive effects of Siglec-9 are mediated by

SHP-1, not SHP-2 (415). Since only the later became activated (Figure 6.5), it is possible that Siglec-9 engagement did not induce any endocytotic functions. On the other hand Siglec-5 ITIM phosphorylation is independent of inhibitory signalling (254).

It is worth noting that binding of *C. jejuni* 11168 to Sn would have an opposing effect on phagocytosis as Sn activation enhances the phagocytosis of *C. jejuni* in macrophages (394),(395). Furthermore, human Siglecs are expressed in pairs of two, where receptors share nearly identical extracellular ligand-binding regions, yet have divergent trans-membrane and cytoplasmic regions. The inhibitory ITIM-containing Siglec-5 is paired with the activating ITAM-containing Siglec-14 (420) to counter pathogen exploitation of inhibitory signalling by providing the host with additional activatory pathways as research on GBS has recently shown (416). It is currently unknown whether *C. jejuni* 11168 can activate Siglec-14 and potentially modulate host immune response through this interaction. These reports outline the complex signalling properties of Siglec activation and further studies are required to decipher the interplay of downstream mediators triggered by *C. jejuni* infection.

Prior work from Dr Holly Stephenson had demonstrated the capacity of *C. jejuni* 11168 to bind another Siglec receptor; Siglec-10 (218). Using CHO cells overexpressing the Siglec-10 receptor, the study demonstrated a significant increase in binding of WT *C. jejuni* to Siglec-10 expressing CHO cells. Importantly, this increase was not noted in response to the ΔflaA or the Pse-negative ΔCj1316 isogenic mutant strains, indicating that binding is mediated by Pse structures on the flagellum surface (218). As detailed in Chapter 5, Pse structures on the flagellum were able to modulate the IL-10 response of dTHP-1 cells to *C. jejuni* 11168. Here, we have identified a link between the engagement of Siglec-10 and the enhancement of IL-10 production by the flagellum. Firstly, we established the presence of Siglec-10 in human M1- and M2-macrophages

(Figure 6.1) and observed up-regulation of Siglec-10 expression following differentiation of THP-1 cells (Figure 6.2), similar to reports that Siglec-9 becomes up-regulated during the development of monocytes into immature DCs (421). Critically, over-expression of the receptor in THP-1 cells led to an increase in flagellum-mediated enhancement of IL-10 (Figure 6.4). It is important to note that *C. jejuni*-mediated IL-10 induction is predominantly TLR/MyD88-dependent (218) and Siglec-10 engagement modulates this TLR-signalling to enhance the production of IL-10. While this is the first report of Siglec-10 mediated modulation of IL-10, both Siglec-5 and Siglec-9 have been linked to host IL-10 immunity as discussed earlier. In a similar approach, receptor over-expression in THP-1 cells led to 2.5 - 4 times higher IL-10 production upon stimulation with LPS or PGN (401).

As mentioned above, we did not detect phosphorylation of the Siglec-10 ITIM domain in dTHP-1 cells co-cultured with *C. jejuni* (Figure 6.5). However, ITIM activation would lead to modulation of pro-inflammatory signalling (260), which is unaffected by the flagellum (see Chapter 5). Despite ITIMs being the best characterised intracellular signalling Siglec domains, Siglec-10 also contains a putative growth factor receptor binding protein-2 (Grb2) binding motif (422). Grb2-mediated signalling is known to alter p38 signalling (423), one of the main pathways involved in *C. jejuni*-mediated IL-10 production (see Chapter 5). Future studies must identify how Siglec-10 engagement may modulate activation of p38. Sotomayer and colleagues have previously reported that the flagellin moiety of *Salmonella* typhmurium is a negative regulator of LPS-mediated IL-10 expression; and this cellular process is TLR5-dependent (345). Our findings highlight an opposing paradigm whereby the flagellum of *C. jejuni* engages with the host in a TLR5-independent, Siglec-10-mediated manner leading to positive regulation of IL-10. In conjunction with the evidence of Siglec-5 and Siglec-9 activation,

our work has identified *C. jejuni* as a pathogen “geared” towards induction of anti-inflammatory signaling.

Chapter 7

Discussion

There is a host of evidence to suggest commensal, nonpathogenic and pathogenic microorganisms have evolved mechanisms to modulate host immunity. Commensals generally lack the virulence factors found in pathogenic species and therefore do not trigger an inflammatory response (4). Bacterial pathogens, particularly those invading tissues after mucosal colonization, disrupt the intestinal balance and cause overt inflammation involving massive activation of humoral and cellular immune effectors. Naturally, bacterial pathogens have evolved sophisticated mechanisms to suppress inflammation in order to gain advantage and enhance their chances of survival (12). While disruption of tolerance by these organisms has been extensively studied, little is currently known about how they evade or subvert the immune response (7). Some of the most common strategies shared by many pathogens is modulation of their surface and antigenic hypervariability in order to avoid recognition or activate inhibitory receptors in immune cells.

In this work we have identified the role of microbial motifs from the two most predominant gastrointestinal pathogens in the evasion on host immunity. We hypothesised that the structural components of *C. difficile* and *C. jejuni* facilitate immune evasion by engaging inhibitory receptors on innate immune cells. Firstly, we showed that PGN from *C. difficile* activates the tolerogenic receptors NOD1 and NOD2, without triggering activation of the inflammasome. PGN was also able to modulate TLR signaling in response to other MAMPs, altering the cytokine response of human monocytes. Furthermore, while the acute cytokine response to LPS was enhanced in the presence of PGN, chronic stimulation with PGN was able to alter the fate of MDMs and diminish their response to LPS stimulation. Naïve immune cells exposed to PGN adopted a more tolerogenic profile during maturation as evidenced by the expression of surface molecules and secretion of cytokines. Given the prevalence of *Clostridiales* in the gut, we can expect PGN fragments released in the environment to inform the development of APCs. A recent study showed in vivo immunomodulation by NOD1 ligands could affect neutrophil function in mice by priming the innate immune system (311). More *in vivo* experiments are needed to verify the physiological relevance of these findings as the majority of current evidence are drawn from *in vitro* studies. Finally, it was interesting to observe the difference in TLR activation by and cytokine response to PGN polymers from *C. difficile* and *S. aureus*, suggesting the bacteria may have adapted their surface antigens to suit their colonisation strategy.

We then concentrated on *C. jejuni*, which has evolved mutations in its flagellum such

that it no longer interacts with TLR5 (217). Instead, *C. jejuni* flagella activate the immunoinhibitory Siglec-10 receptor to induce production of IL-10 (203). We studied the mechanism by which selective enhancement takes place and showed that flagella can specifically activate signaling mediators that control IL-10 expression. Although, the anti-inflammatory properties of IL-10, including its ability to prevent *C. jejuni*-mediated colitis, have been described elsewhere (382), we have demonstrated that *C. jejuni* has evolved surface motifs that will engage receptors linked IL-10 induction. It will be interesting to study whether other proteobacteria have evolved to exploit similar cellular mechanisms.

Interestingly, we were able to demonstrate the novel interaction of *C. jejuni* with two further Siglec receptors; Siglec-5 and Siglec-9. Both have been involved in the inhibition of phagocytosis, dampening of TLR signaling and blocking of pro-inflammatory cytokine secretion. The interaction of *C. jejuni* to Siglec-9 was dependent on the Neu5Ac SA of LOS, which exhibits molecular mimicry to host gangliosides (196). Neu5Ac is the predominant SA found on mammalian cells and many human pathogens, including *C. jejuni*, coat their surface with it. Presumably, mimicry of host glycans is a potential immune evasion strategy, as immune cells with specificities for these glycans will be deleted leading to increased peripheral tolerance. A similar mechanism of bacterial immune evasion was reported for GBS, which can impair neutrophil defense functions by coopting a host inhibitory receptor via sialoglycan molecular mimicry (264). The functional outcome of *C. jejuni* engagement with the two receptors has yet to be determined.

Furthermore, it is thought that epitopes shared by bacteria and host tissues may contribute to autoimmunity, such as the development of Guillain-Barré syndrome by patients with campylobacteriosis. Therefore, the study of these interactions may not only contribute to the fight of acute infections but also chronic diseases. Overall, we have presented evidence that pathogenic bacteria have evolved surface motifs which can engage immunoinhibitory receptors in order to dampen inflammation. This suggests that survival, and not just pathogenesis, has been an important part in the evolution of the most successful pathogens. The study of immune manipulation by bacteria is an emerging field that offers a promising new interface in microbiology and immunology.

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